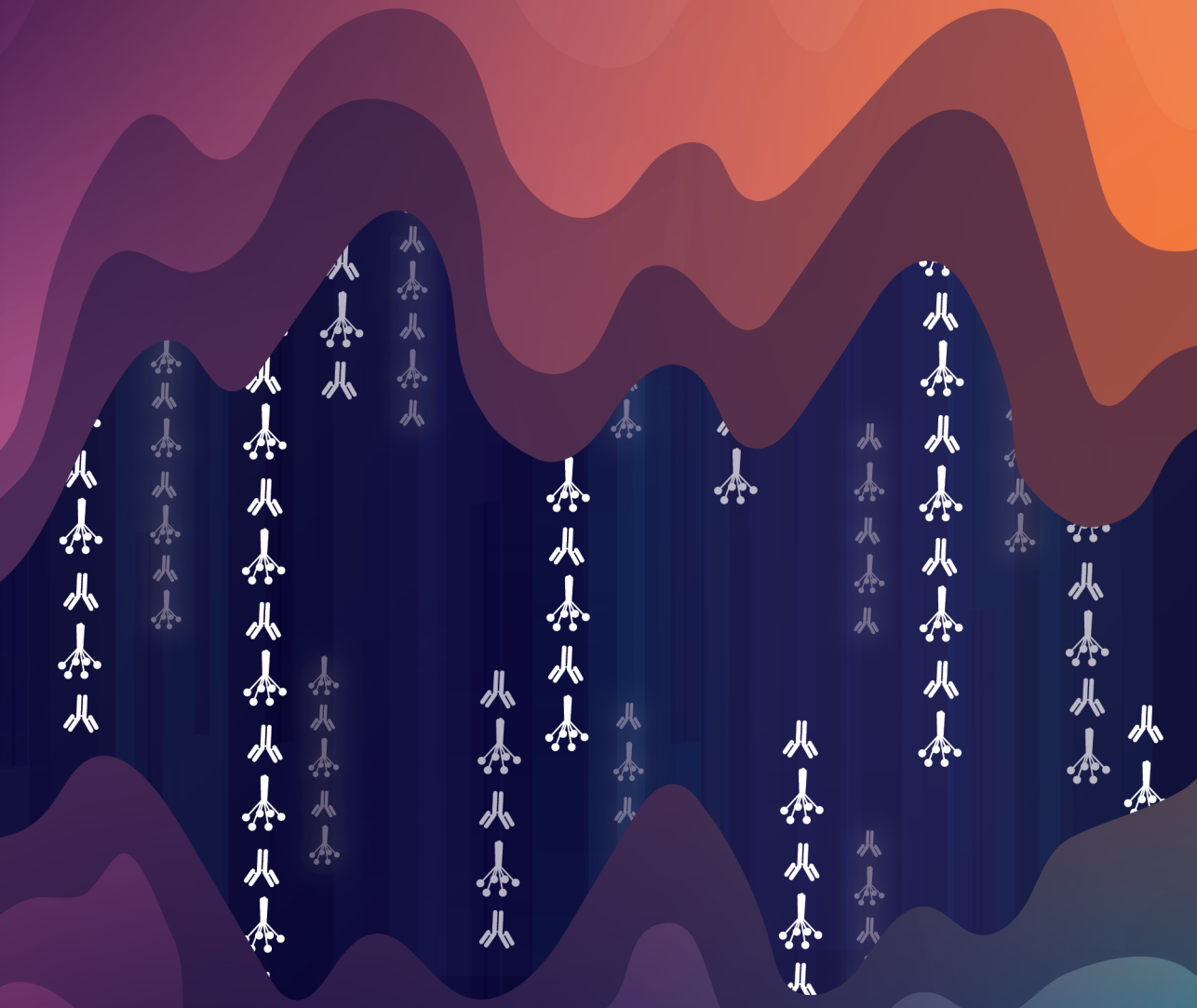
C1q and anti-C1q autoantibodies in (auto)immunity

Dijkstra, D.J.

C1q and anti-C1q autoantibodies in (auto)immunity



Douwe J. Dijkstra

C1q and anti-C1q autoantibodies in (auto)immunity

Douwe J. Dijkstra

Cover design: Marloes Grobben
Layout: Ridderprint | www.ridderprint.nl
Printing: Ridderprint | www.ridderprint.nl
ISBN: 978-94-6483-982-1

The research described in this thesis was performed at the Department of Immunology of the Leiden University Medical Center in Leiden, the Netherlands.

Financial support for the printing of this thesis was kindly provided by Hycult Biotech and Svar Life Science.

C1q and anti-C1q autoantibodies in (auto)immunity

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 28 mei 2024
klokke 10.00 uur

door

Douwe Jan Dijkstra
geboren te Groningen
in 1993

Promotores

Prof. dr. L.A. Trouw

Prof. dr. P.W.H.I. Parren

Leden promotiecommissie

Prof. dr. C. van Kooten

Prof. dr. A.C. Lankester

Dr. S.A. Joosten

Prof. dr. J.H.W. Leusen Universiteit Utrecht

Prof. dr. G. Vidarsson Universiteit Utrecht

TABLE OF CONTENTS

CHAPTER 1

General introduction

Douwe J. Dijkstra

The human immune system

The immune system is a major contributor to human health by protecting against various threats from both inside and outside the body. Humans are constantly exposed to pathogens like bacteria and viruses. However, the immune system is usually able to quickly eliminate these pathogens before we get very sick. The importance of our immune system is underscored by the fact that large parts of it are evolutionarily conserved among mammals and to a degree among other jawed vertebrate species [1]. The human immune system consists of both cellular and humoral (proteins and other molecules secreted by both immune and non-immune cells) components.

The human immune system can be divided into two parts: the innate part and the adaptive part. While these two parts heavily supplement and even depend on each other, they fundamentally differ in their mode of operation. The innate immune system comprises multiple cell types and sets of proteins (such as the complement system), which can react very fast to any threat, are often short-lived, and do not have a 'memory' of past encounters with pathogens [2]. Although there are exceptions to all of these characteristics, the cells and molecules of innate immunity can be seen as first responders, they start acting within seconds to minutes of the appearance of a potential threat. They are important in clearing the bulk of pathogens, but also dead cells and debris, and are mainly generalists which rely on a limited set of pattern recognition receptors to identify their target [3]. Upon recognition and elimination of pathogens, innate immune cells can also communicate with each other, through the release of cytokines to either enhance or decrease the local state of inflammation and immune activation. This process is especially important for the activation and instruction of the adaptive immune system.

The adaptive or acquired immune system takes a longer time (in the order of days up to one or two weeks) to develop a full response upon the first encounter, which will then be very specific for the current threat. The main cell types in adaptive immunity are B and T lymphocytes, of which there is a continuous generation of a pool of naïve cells. Each of these cells have a different receptor with which they recognize a specific target, their antigen. When these B and T cells encounter their antigen, they require an extra stimulatory signal instructing them to proliferate and specialize, from a combination of other activated immune cells, the activated complement system or other danger signals [4]. This instruction of adaptive immune cells by the innate immune system is an important example of the communication between the two different parts of the immune system. However, the interaction can also be in the other direction, from

adaptive to innate, for instance when immunoglobulins produced by B cells bind their antigen and instruct complement and innate cells to react against this antigen.

When a particular threat (like an infection) has passed, it is crucial that the cellular immune response contracts and the inflammation stops. At the same time, some cells of the adaptive immune system, so-called memory cells, continue to exist for years and have the ability to quickly proliferate upon re-infection. The retaining of memory cells helps the adaptive immune elicit a faster and stronger response upon the next encounter of the same pathogen and thereby provides better protection. This process is exploited in the case of vaccination. A vaccine typically contains a dead bacterium or virus, or one of its components, to mimic an infection, and to which the body raises an initial immune response including memory cells. Subsequently, when an actual infection with this particular pathogen occurs, there are already specific B and/or T cells in place to quickly reactivate and respond against the infection.

The complement system

A collection of interacting proteins called the complement system forms an integral part of the innate immune system. While the complement system was initially named because it 'complemented' immunoglobulins in the killing of bacteria, many additional functions for complement have since been described [5]. The complement system consists of the classical, lectin and alternative pathways, which all converge into the terminal pathway. The chain of activating molecules starting from the classical pathway are termed C1 to C9, while cleaved products are denoted with the addition of an 'a' or 'b' [6]. Various inhibitors, which can be found both in fluid phase and on the surface of cells, act on most of the intermediate molecules to regulate these activation pathways. In this thesis, there is much focus on C1q, which is the recognition protein for the classical pathway. It consists of six arms with an N-terminal collagen-like region (CLR) and a C-terminal globular head (GH), which are linked at the CLR. Each arm is a heterotrimer of polypeptides (A, B and C chains) connected through disulfide bonds. The elaborate architecture of 18 polypeptide chains gives C1q its large size of approximately 450 kDa and it is abundantly present in the circulation, with a serum concentration around 0.2 mg/ml. While the majority of complement proteins are synthesized in the liver, C1q is, in contrast, produced by several types of immune cells, mainly monocytes, macrophages and dendritic cells [7].

The GHs of C1q can bind to various isotypes of immunoglobulins, C-reactive protein (CRP) and dead or dying cells, and thereby allow its associated enzymes C1r and C1s

to cleave C4 and C2. The activated fragments C4b and C2a together deposit on the cell surface of the target and form the convertase that activates C3. Similarly, the lectin pathway is initiated by mannose-binding lectin (MBL), collectins or ficolins, which also use C4 and C2 to activate C3 and the further complement cascade. Addition of a cleaved C3b to C4b and C2a completes the convertase which activates C5. The alternative pathway is constitutively activated by the spontaneous hydrolysis of C3, which binds factor B and allows factor D to cleave and activate another molecule of C3 into C3b and C3a and thereby continuously challenges defense by complement regulators on the cells in its immediate environment. Foreign cells and particles are not protected by human complement regulatory proteins and will therefore be subject to alternative pathway complement activation. Because the deposition of C3b and subsequent recruitment of factor B can cause the activation of yet more C3, the alternative pathway also serves as an amplification loop for the classical and lectin pathways.

After the activation pathways converge at C3 activation, the complement cascade reaches its end when the complement proteins C5b, C6, C7, C8 and 18 copies of C9 form the membrane attack complex (MAC), thereby puncturing the cell membrane, aimed at causing cell lysis [8]. Apart from direct lysis by the MAC, the deposition of activated complement components, especially C3b, activates immune cells with complement receptors to act against the opsonized target. Both the complement system as a whole, and individual proteins outside the context of complement activation, also have functions other than defense against pathogens [9]. For example, when antibodies bind their target and form immune complexes in the blood, complement deposition allows their clearance from the circulation through interaction with complement receptor 1. Similarly, binding of C1q and a controlled amount of complement activation on dying or dead cells can be beneficial. It helps phagocytes in their waste disposal task via complement receptors, while moderation is needed to prevent formation of the MAC and spilling of cellular content into the environment [10]. Deficiency of C1q or other classical complement pathway proteins is associated with inadequate clearance of cellular debris, and often leads to autoimmune disease, which will be discussed later. This adds to the status of the complement system as an indispensable component of healthy human physiology.

B cells and antibodies

The human adaptive immune system has three major classes of molecules to recognize antigens: antibodies (also called immunoglobulins; Ig), T-cell receptors and the major histocompatibility complex (MHC, also called human leukocyte antigen; HLA) proteins

[4]. Of these, antibodies are able to bind the broadest range of antigens, with proteins as well as lipids, polysaccharides and small chemicals as possible targets, and have the highest potential binding strength. Antibody classes are split into five isotypes based on the structure of their HC constant domains: IgA, IgD, IgE, IgG and IgM. Of these, IgA can be further divided into subclasses IgA1 and IgA2, while IgG similarly consists of subclasses 1 to 4. IgG is the most abundant isotype in human blood, and is the focus of antibody research in this thesis. IgG antibodies are produced as a symmetric molecule of four peptide chains which are linked by disulphide bonds: two identical heavy chains (HC) and two identical light chains (LC) (Figure 1). Other isotypes generally follow a similar architecture, although modification and/or multimerization occur for certain isotypes. Each LC consists of one variable domain and one constant domain, which can be either of the kappa or lambda type. An HC also contains one variable domain, but three or four constant domains, depending on the isotype. The LC together with the variable domain and first (counting from the N terminus) constant domain of the HC form the 'Fragment, antigen binding' (Fab), while the further constant domains of the two HCs form the 'Fragment, crystallizable' (Fc) [11]. Both Fabs are linked to the Fc at the hinge region, which is also the site where the two HCs are covalently linked through disulphide bonds. The specificity of an antibody is determined by the composition of the variable domains, particularly by the complementarity determining regions (CDRs) within. These CDRs contain the amino acids that come into contact with the antigen, the part of the antigen which interact with the antibody is called epitope.

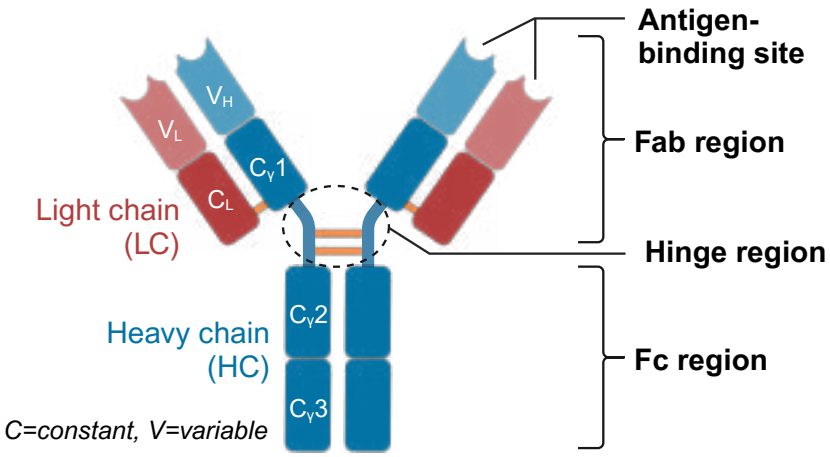


Figure 1. Overview of the major structural components of antibodies, represented by IgG, which is the most abundant isotype in human blood. Created with BioRender.com

The production of antibodies is performed by plasma B cells, whose progenitors originate from stem cells in the bone marrow. When these cells differentiate into pre-B cells, their specificity is determined for the first time. In stem cells, the germline DNA sequence from which the variable domains of antibodies will later be made contain many V, D and J genes, but pre-B cells randomly select one of each in a process called V(D)J recombination [12]. This process occurs separately for the HC and the LC, and may be repeated if an unproductive rearrangement has been made. The HC locus contains about 45 functional V genes, 23 D genes and 6 J genes, giving rise to a large number of unique combinations and large diversity between HCs. The LC locus does not have D genes, but contains 35 V genes and 5 J genes for kappa LC, or 30 V genes and 4 J genes voor lambda LC, multiplying the number of unique HC-LC pairings even further [4]. Another large contributing factor to diversity among antibodies is the addition or deletion of nucleotides at the junctions between V, D and J genes (or between V and J for the LC) during the recombination process, leading to billions of possible clones. When these steps have occurred, the cell has become an immature B cell, with a unique specificity which it expresses as a membrane-bound immunoglobulin molecule, the B-cell receptor. These cells then leave the bone marrow to mature in peripheral lymphoid tissue, such as lymph nodes and the spleen.

Most naïve B cells are follicular B cells, which travel though the blood stream from one lymphoid organ to another until it encounters an antigen. When a B cell recognizes an antigen with its B-cell receptor, other receptors on the B cell can provide extra stimulation (Figure 2A). This can be facilitated for instance by complement receptor 2 binding to deposited C3 fragments on the antigen, or by Toll-like receptors binding their targets of bacterial or viral origin [13]. Activation of these receptors functions like an additional verification that an antibody response against this particular antigen is indeed desirable. In some cases, especially with non-protein antigens which are multivalent, B cells can activate and proliferate without the help of other cells [14]. However, responses to most proteins require the stimulation of helper T cells. A protein antigen bound by the B-cell receptor is internalized, processed by the internal machinery and then presented in the form of peptide fragments in the MHC class II molecules on the cell membrane [15]. An activated helper T cell recognizes specific peptides embedded in MHC class II and provides a stimulating signal to the B cell via membrane proteins such as CD40 (on the B cell) and CD40 ligand (on the T cell), as well as cytokines produced by the helper T cell [16]. Development of an antibody response therefore requires not only a B cell recognizing the antigen, but often also a specific T cell.

Activated B cells migrate into the lymphoid follicles of lymphoid organs to form a germinal center, where they rapidly proliferate. Under stimulation from specialized follicular T

helper cells and follicular dendritic cells, B cells undergo the so-called germinal center reaction, including affinity maturation and isotype switching. Isotype switching consists of a DNA recombination event where the B cell switches to produce another constant domain for the HC [17]. Affinity maturation is caused by somatic hypermutation, a process where variable domain-encoding DNA in proliferating B cells is subject to an exceptionally high rate of mutation [18]. When this process leads to a higher affinity B-cell receptor, this clone has favorable competition for antigen capture and T cell stimulation, and therefore a higher chance of survival and further proliferation. After undergoing the germinal center reaction, B cells differentiate into either long-lived plasma cells, which produce antibody while residing in the bone marrow, or into circulating memory cells [19]. B cells can also be stimulated extrafollicularly, where limited isotype switching occurs and short-lived plasma cells are generated.

The antibodies produced by B cells after a germinal center reaction have high avidity for their antigen and are generally of an isotype other than IgM. For their role in immune defense, antibodies rely on several effector functions (Figure 2B), which may be altered by structural modification of the antibody [20]. Firstly, binding to a microbial antigen may allow neutralization of this microbe by blocking critical surface components or neutralization of toxins [21]. Secondly, the Fc domain of antibodies is the ligand for Fc receptors on various immune cells. Antibodies have varying avidities for the different types of Fc receptor depending on isotype and subclass [22]. Through Fc receptors, antibodies may allow phagocytosis by phagocytes, or antibody-dependent cellular cytotoxicity by natural killer cells, granulocytes or macrophages. Lastly, clustered Fc domains of most IgG subclasses and IgM also form a ligand for C1q after a conformational change upon antigen binding [23]. Activation of the complement system may result in direct lysis of the targeted microbe, opsonization with deposited complement fragments and stimulation of inflammation. These effector functions benefit greatly from enhanced avidity by binding antigens with multiple Fab arms. Furthermore, interactions between the Fc regions of antibodies allow clustering upon antigen binding, thereby further increasing total avidity for the antigen and for several receptors and C1q [24]. Antibodies of the most common isotype in blood, IgG, can remain in the circulation for a long time, with a half-life up to 3 weeks for most IgG subclasses. To accomplish this, IgG binds to the neonatal Fc receptor, which rescues IgG from degradation in lysosomes and recycles it to the cell surface. The combination of these effector functions, the broad range of possible targets and the capacity for high avidity and specificity make antibodies a potent part of the immune system, and a highly-explored option for therapeutics, of which already over 100 have been approved.

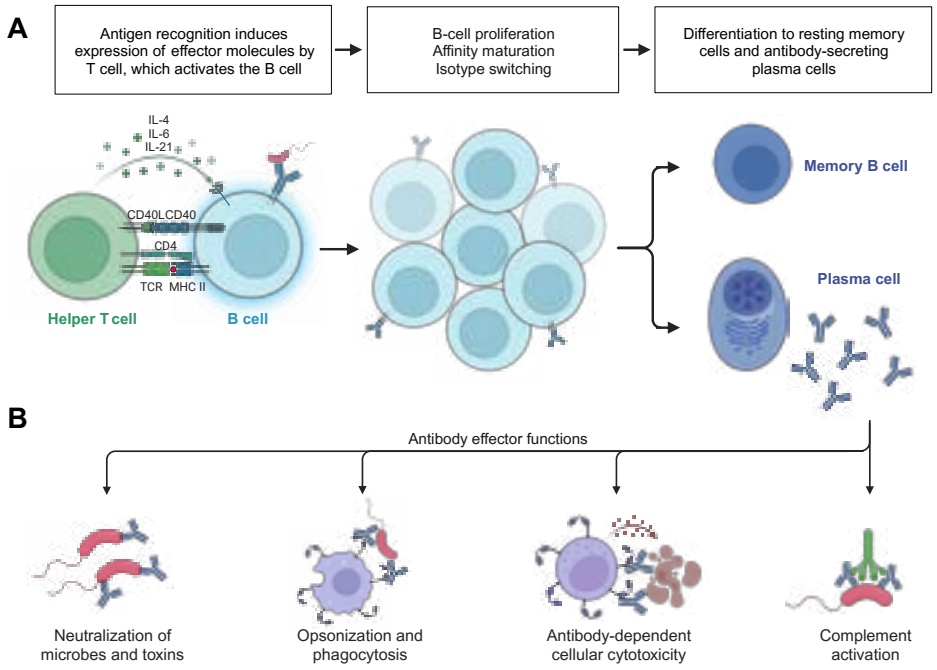


Figure 2. B cell development and antibody effector functions. (A) In addition to recognizing their antigen, B cells must receive stimulatory signals, often from helper T cells, in order to be activated. They can then enter a germinal center, in which they mature their specificity and switch antibody production to a new isotype. These B cells can differentiate into long-lived memory cells, or antibody-producing plasma cells. (B) Antibodies have various effector functions, each with varying effectiveness per antibody isotype. Created with BioRender.com

Tolerance, autoimmunity and autoantibodies

As the immune system is capable of mounting a strong and aggressive response to a wide range of antigens, it is of great importance that all molecules produced by the organism itself are tolerated. In the case of both B cells and T cells, antigen receptors with a large repertoire of specificities are generated through random events like recombination and mutation. Sometimes, these antigen receptors may turn out to recognize a self-antigen, causing a risk of attacking the body's own tissues. Therefore, when a naïve lymphocyte in its generative lymphoid organ recognizes a self-antigen, it either undergoes apoptosis, edits its receptor to no longer recognize the self-antigen (in the case of B cells), or develops into a regulatory cells (in the case of some T cells) [25]. As opposed to this central tolerance mechanism for naïve lymphocytes during their maturation, peripheral tolerance can be formed in mature lymphocytes. When mature lymphocytes recognize their antigen in the absence of stimulating or inflammatory signals, they die by apoptosis or enter a state of anergy, where they are no longer

responsive to their antigen [26]. Additionally, peripheral tolerance is maintained by regulatory T cells, which suppress lymphocytes specific to self-antigens.

Under certain conditions, a break in the tolerance towards self-antigens may occur and the immune system may react against autologous antigens. The recognition of self-antigens by the immune system is called autoimmunity, and when this has pathogenic effects and causes symptoms, it is termed autoimmune disease. Autoimmune diseases can be either systemic or local depending on the distribution of the self-antigen, and are often chronic due to the continued availability of antigen and the amplification mechanisms in the activated immune system. While the exact cause of autoimmune diseases is often difficult to pinpoint, they are generally ascribed to certain genetic risk factors, environmental factors and triggers of immunity, such as infections or tissue injury [27, 28]. Inadequate disposal of waste and cellular debris can cause exposure to DNA, histones and other intracellular antigens which are spilled from these cells. These self-antigens may provide a target for autoreactive lymphocytes which would not be available under physiologic conditions.

In this thesis, the most prominent aspect of autoimmunity is the occurrence of antibodies against autologous proteins, so-called autoantibodies. These autoantibodies result from B cells which have escaped the regulatory pathways of the immune system that control self-antigen recognition. This may be because central tolerance failed to eliminate an autoreactive naïve B cell, or because a B cell originally recognizing a (slightly) different antigen matured and developed cross-reactivity to a self-antigen. One type of autoantibody, which is discussed in several chapters of this thesis, recognizes C1q. Anti-C1q autoantibodies occur in a few percent of the general population, meaning that a breach in the tolerance to this self-antigen is relatively common. However, anti-C1q is also strongly associated with the occurrence and severity of kidney injury (lupus nephritis) in patients with systemic lupus erythematosus [29]. One reason that anti-C1q autoantibodies are relatively common may be that they specifically recognize solid-phase C1q (Figure 3). When C1q binds one of its ligands, like a cluster of IgG Fc domains, it docks on to this ligand with its six globular heads. In this process, a conformational change occurs in the C1q molecule, C1q in this state is called solid-phase C1q [30]. The conformational change makes the epitope of anti-C1q autoantibodies available [31]. Due to this property, C1q and its autoantibody do not interact in the solution, but only where ligands of C1q are present. For this reason, it is possible for individuals to have anti-C1q autoantibodies and C1q abundantly present in the blood, while remaining healthy.

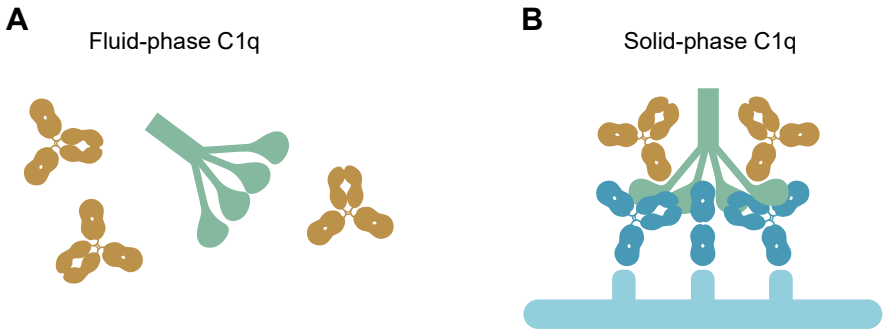


Figure 3. C1q can exist in multiple conformational states, which determines whether anti-C1q autoantibodies will bind. (A) When C1q is in solution, not bound to any ligand, the epitope of anti-C1q autoantibodies is not available. (B) Upon binding to a ligand such as a hexameric cluster of IgG antibodies, C1q changes conformation. The epitope for anti-C1q autoantibodies now becomes available and the autoantibodies bind to this 'solid-phase' C1q.

Scope of this thesis

This thesis investigates multiple facets of the complement system, specifically the classical pathway initiator C1q, and autoantibodies to complement proteins in various diseases. **Chapter 2** reviews the different roles complement activation and regulation can play in the rheumatic diseases systemic lupus erythematosus, rheumatoid arthritis and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Previous research has identified C1q as a possible biomarker for active lung infection by tuberculosis bacteria, **chapter 3** confirms and builds on this. This work investigates the value of C1q as a diagnostic tool for active tuberculosis infection and specifically uveitis complications.

In **chapter 4**, we continue to examine C1q, but also alternative complement pathway regulator Factor H and autoantibodies against these complement proteins. The setting of this chapter is pregnancy, and the common pregnancy complication preeclampsia. Anti-C1q autoantibodies are also the subject of **chapter 5**, where we find that the presence of these autoantibodies is not fit as a biomarker for involvement of the lung in systemic sclerosis. Subsequently, **chapter 6** reports on the isolation of human anti-C1q autoantibodies and their characterization on a monoclonal level, which may help to better understand their involvement in disease. Finally, **chapter 7** summarizes and discusses the results and impact of the research presented in this thesis, and relates it to the available literature.

References

1. M. F. Flajnik, M. Kasahara, Origin and evolution of the adaptive immune system: Genetic events and selective pressures. *Nat Rev Genet* **11**, 47-59 (2010).
2. B. P. Kaur, E. Secord, Innate immunity. *Immunol Allergy Clin North Am* **41**, 535-541 (2021).
3. R. Medzhitov, Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819-826 (2007).
4. A. K. Abbas, A. H. Lichtman, S. Pillai, *Cellular and molecular immunology* (Elsevier Saunders, 2014).
5. P. F. Zipfel, C. Skerka, From magic bullets to modern therapeutics: Paul ehrlich, the german immunobiologist and physician coined the term 'complement'. *Mol Immunol* **150**, 90-98 (2022).
6. M. J. Walport, Complement. First of two parts. *N Engl J Med* **344**, 1058-1066 (2001).
7. R. Lubbers, M. F. van Essen, C. van Kooten, L. A. Trouw, Production of complement components by cells of the immune system. *Clin Exp Immunol* **188**, 183-194 (2017).
8. M. Serna, J. L. Giles, B. P. Morgan, D. Bubeck, Structural basis of complement membrane attack complex formation. *Nat Commun* **7**, 10587 (2016).
9. D. Ricklin, G. Hajishengallis, K. Yang, J. D. Lambris, Complement: A key system for immune surveillance and homeostasis. *Nature immunology* **11**, 785 (2010).
10. A. J. Nauta, L. A. Trouw, M. R. Daha, O. Tijsma, R. Nieuwland, W. J. Schwaeble, A. R. Gingras, A. Mantovani, E. C. Hack, A. Roos, Direct binding of c1q to apoptotic cells and cell blebs induces complement activation. *European journal of immunology* **32**, 1726-1736 (2002).
11. G. Vidarsson, G. Dekkers, T. Rispens, Igg subclasses and allotypes: From structure to effector functions. *Front Immunol* **5**, 520 (2014).
12. D. G. Schatz, Y. Ji, Recombination centres and the orchestration of v(d)j recombination. *Nat Rev Immunol* **11**, 251-263 (2011).
13. M. J. Walport, Complement. Second of two parts. *N Engl J Med* **344**, 1140-1144 (2001).
14. F. A. Bonilla, H. C. Oettgen, Adaptive immunity. *J Allergy Clin Immunol* **125**, S33-40 (2010).
15. M. I. Yuseff, P. Pierobon, A. Reversat, A. M. Lennon-Duménil, How b cells capture, process and present antigens: A crucial role for cell polarity. *Nat Rev Immunol* **13**, 475-486 (2013).
16. J. E. Craft, Follicular helper t cells in immunity and systemic autoimmunity. *Nat Rev Rheumatol* **8**, 337-347 (2012).
17. J. Stavnezer, J. E. Guikema, C. E. Schrader, Mechanism and regulation of class switch recombination. *Annu Rev Immunol* **26**, 261-292 (2008).
18. M. S. Neuberger, Antibody diversification by somatic mutation: From burnet onwards. *Immunol Cell Biol* **86**, 124-132 (2008).
19. T. Inoue, T. Kurosaki, Memory b cells. *Nat Rev Immunol* 10.1038/s41577-023-00897-3 (2023).
20. T. Damelang, M. Brinkhaus, T. L. Van Osch, J. Schuurman, A. F. Labrijn, T. Rispens, G. Vidarsson, Impact of structural modifications of igg antibodies on effector functions. *Frontiers in Immunology* **14**, 1304365 (2024).

21. D. R. Burton, Antiviral neutralizing antibodies: From in vitro to in vivo activity. *Nat Rev Immunol* **23**, 720-734 (2023).
22. P. Bruhns, B. Iannascoli, P. England, D. A. Mancardi, N. Fernandez, S. Jorieux, M. Daëron, Specificity and affinity of human fcgamma receptors and their polymorphic variants for human igg subclasses. *Blood* **113**, 3716-3725 (2009).
23. D. Ugurlar, S. C. Howes, B.-J. de Kreuk, R. I. Koning, R. N. de Jong, F. J. Beurskens, J. Schuurman, A. J. Koster, T. H. Sharp, P. W. Parren, Structures of c1-igg1 provide insights into how danger pattern recognition activates complement. *Science* **359**, 794-797 (2018).
24. S. C. Oostindie, G. A. Lazar, J. Schuurman, P. Parren, Avidity in antibody effector functions and biotherapeutic drug design. *Nat Rev Drug Discov* **21**, 715-735 (2022).
25. D. Nemazee, Receptor editing in lymphocyte development and central tolerance. *Nat Rev Immunol* **6**, 728-740 (2006).
26. D. L. Mueller, Mechanisms maintaining peripheral tolerance. *Nat Immunol* **11**, 21-27 (2010).
27. M. H. Cheng, M. S. Anderson, Monogenic autoimmunity. *Annu Rev Immunol* **30**, 393-427 (2012).
28. A. V. Chervonsky, Influence of microbial environment on autoimmunity. *Nat Immunol* **11**, 28-35 (2010).
29. F. S. van de Bovenkamp, D. J. Dijkstra, C. van Kooten, K. A. Gelderman, L. A. Trouw, Circulating c1q levels in health and disease, more than just a biomarker. *Mol Immunol* **140**, 206-216 (2021).
30. S. Uwatoko, S. Aotsuka, M. Okawa, Y. Egusa, R. Yokohari, C. Aizawa, K. Suzuki, C1q solid-phase radioimmunoassay: Evidence for detection of antibody directed against the collagen-like region of c1q in sera from patients with systemic lupus erythematosus. *Clin Exp Immunol* **69**, 98-106 (1987).
31. M. D. Golan, R. Burger, M. Loos, Conformational changes in c1q after binding to immune complexes: Detection of neoantigens with monoclonal antibodies. *J Immunol* **129**, 445-447 (1982).

CHAPTER 2

Complement activation and regulation in rheumatic disease

Douwe J. Dijkstra[1], Jivan V. Joeloemsingh[1], Ingeborg M. Bajema[2], Leendert A. Trouw[1]

1. Department of Immunology, Leiden University Medical Center, Leiden, the Netherlands
2. Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands

Seminars in Immunology (2019), doi: *10.1016/j.smim.2019.101339*

Abstract

Complement is a key component of the innate immune defense and in addition forms a bridge to the adaptive immune responses. As such complement is of vital importance for efficient protection against infections. However, the activity of the complement system can also aberrantly be directed against the tissues of the body itself and contribute to organ damage in a variety of diseases. In several rheumatic diseases complement activation is suggested to play a pronounced role. This review will highlight the role of both complement activation and complement regulation in rheumatic disease. A contribution of complement to the disease process is often suggested based on the presence of complement activation fragments in the target tissues or the presence of complement activation fragments in the circulation. The role that complement plays in different rheumatic diseases is often unknown but is thought to contribute to tissue damage as a consequence of autoantibody mediated immune complex formation and deposition. In addition reduced complement inhibition mediated by endogenous complement regulators can also enhance complement activity and tissue damage. In observational studies, it is difficult to distinguish whether complement activation is a result of enhanced activation or decreased regulation. Until recently, strong conclusions on the relative importance of complement activation to the pathology were largely restricted to animal experiments. Usage of complement targeting therapeutics in humans will hopefully give us the opportunity to study the actual contribution of complement activation towards disease progression and tissue damage in rheumatic disease into more detail.

Complement activation and regulation

The complement system is one of the oldest parts of the innate immune system and is involved in numerous processes like elimination of microbes, clearance of immune complexes, tissue regeneration and angiogenesis [1]. Unfortunately, while complement is very efficient in protecting against infections, it is also activated against one's own tissue in several autoimmune and rheumatic diseases. Three pathways lead to activation of the complement cascade; the classical pathway, lectin pathway and alternative pathway (Figure 1). All three activation pathways eventually lead to the cleavage of C3 and deposition of C3b, followed by the terminal pathway that results in the formation of the membrane-attack complex (MAC) and the release of anaphylatoxins C3a and C5a.

The classical pathway (CP) of complement activation is initiated when the recognition molecule C1q binds to its ligands such as surface-bound IgM, multimers of IgG, C-reactive protein (CRP), DNA or dead cells [2]. Following the binding of C1q to its targets, the C1 associated enzymes C1r and C1s become active enabling them to initiate complement activity by cleaving C4 and C2, resulting in the formation of the CP C3-convertase C4b2a [3].

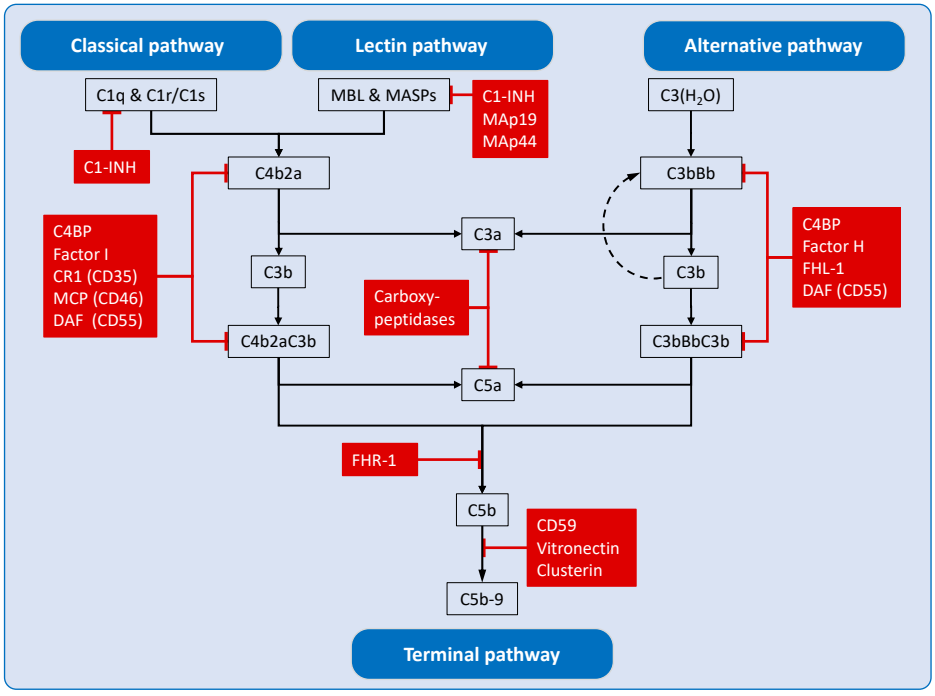


Figure 1. An overview of the complement system, indicating the interactions of activating (black boxes and lines) and regulating (red boxes and lines) complement components.

The lectin pathway (LP) of complement activation is initiated in a similar manner, but by other pattern-recognition receptors (PPRs), like mannose-binding lectin (MBL), ficolins (lectins containing a fibrinogen-like and a collagen-like domain) or collectins (collagenous C-type lectins) [4]. After ligand binding, these recognition molecules activate the LP via the MBL-associated serine proteases (MASPs), which can either activate C4 and C2, leading to the formation of the LP and CP C3-convertase (C4b2a), or directly cleave and activate C3 [5].

The alternative pathway (AP) of complement has a different mechanism of activation. The AP is continuously activated in a spontaneous manner when a small part of the C3 molecules becomes hydrolyzed forming C3(H_2O). C3(H_2O) is bound by factor B and becomes a target for factor D mediated cleavage of factor B. Subsequently, the complex can cleave additional C3 molecules into C3a and C3b. The formed C3b molecules also bind factor B. The subsequent cleavage of bound factor B by factor D forms the AP C3-convertase C3bBb. This process is constantly counteracted on cell surfaces by the endogenous complement inhibitor factor H, which binds polyanionic residues like sialic acid groups and glycosaminoglycans present for instance on human cells. Pathogens often contain fewer of these polyanions, leading to less factor H binding and more complement activation [6]. As such, factor H can be considered the recognition molecule of the AP. The AP is also considered an amplification pathway, as it can be initiated by C3b formed by any of the three pathways [7].

Following the formation of the C3 convertases, the remainder of the complement activation cascade, the terminal pathway, is similar. The C3-convertases form C5-convertases that can cleave C5 into C5b and the potent anaphylatoxin C5a. C5b will, together with C6, C7, C8 and multiple molecules of C9, form the membrane attack complex (MAC) which can cause cell lysis [8]. Most of the circulating complement proteins are produced by hepatocytes, but expression of complement proteins is also recorded for other cell types including leukocytes [9]. Production of several key complement proteins is largely restricted to specialized cell types, for example, factor D is mainly produced by adipocytes and C1q is produced by cells of the monocytic lineage such as macrophages, dendritic cells and mast cells [10-13].

Table 1. An overview of complement regulators, their specific function and references about their role in rheumatic diseases that were consulted in this review.

Name	Full name	Function	References in this review
C1-INH	C1 esterase inhibitor	Inactivates serine proteases C1r, C1s and MASPs	SLE: [110] RA: [70]
MAp19	MBL Associated protein of 19kDa	Competes with MASPs for MBL binding	SLE: [95]
MAp44	MBL Associated protein of 44kDa	Inhibits C4 deposition by binding lectin pathway initiators	RA: [72] SLE: [95]
C4BP	C4b-Binding Protein	Inhibits functions of C4, accelerates decay of C3 convertases	RA: [66, 71] SLE: [28]
Factor H		Binds self surfaces, cofactor for factor I, accelerates decay of convertases	RA: [55-60] SLE: [28, 59, 118, 120, 126] AAV: [137-140]
Factor I		Inactivates C4b and C3b by cleavage, but only after cofactor binding	SLE: [117]
FHL-1	Factor H-like protein 1	Cofactor for factor I, accelerates decay of convertases	RA: [55, 56]
FHR-1	Factor H-related protein 1	Binds self surfaces, inhibits C5 activation and MAC formation	SLE: [119]
CR1 (CD35)	Complement Receptor 1	Cofactor for factor I among other functions (membrane-bound)	SLE: [111, 112]
MCP (CD46)	Membrane Cofactor Protein	Cofactor for factor I (membrane-bound)	SLE: [115, 116]
DAF (CD55)	Decay Accelerating Factor	Accelerates decay of convertases (membrane-bound)	RA: [74-76, 91] SLE: [113, 114]
CD59		Binds C8 and C9, inhibits MAC formation (membrane-bound)	SLE: [113, 114]
Vitronectin		Binds C5b-9, prevents MAC formation	SLE: [80]
Clusterin		Binds C7, C8, C9, prevents MAC formation	SLE: [80]
Carboxypeptidases		Cleaves C3a and C5a to des-arginated forms	RA: [73]

This potentially aggressive and self-amplifying cascade of inflammatory and cytotoxic mediators needs to be tightly regulated to ensure that complement activation is limited in time and location [14]. Excessive and unrestricted complement activation would lead to organ damage and enhanced risk for infections due to the complete consumption of complement. The complement system is therefore regulated by membrane-bound as well as soluble factors (Table 1). There are four general functions of complement inhibitors: some inhibitors bind complement proteins and prevent their incorporation into complement complexes, others bind and terminally inactivate enzymes, other inhibitors serve as a co-factor for enzymatic degradation of complement proteins and other complement inhibitors enhance the decay of C3-convertases [15].

C1-inhibitor (C1-INH) inhibits the classical and lectin pathway by binding and inactivating C1s, C1r and MASPs, whereas MAp19 and MAp44 inhibit the lectin pathway specifically [16, 17]. C4b-Binding Protein (C4BP) inhibits the activity of C4 and accelerates the decay of the C3 convertases of all three pathways [18]. Decay of C3 convertases is also stimulated by factor H, which in addition serves as a cofactor for factor I mediated degradation of C3b [6]. Factor I inactivates C4b and C3b by cleaving them, but only when they are first bound by a cofactor of factor I, such as factor H, factor H like protein 1 (FHL-1), C4BP, MCP (CD46) and complement receptor 1 (CD35). In the terminal pathway, clusterin and vitronectin bind the proteins that form the C5b-9 complex and prevent its completion or insertion into the membrane. A similar function is attributed to CD59 expressed on the cell surface, which binds C8 and C9 to hinder the assembly of the MAC. Another membrane-bound complement regulating protein, decay-accelerating factor (DAF; CD55), increases the degradation of convertases on the cell membrane. Lastly, carboxypeptidase-N converts the potent anaphylatoxins C3a and C5a into their less active, des-arginated forms.

Complement regulation also plays a role in the process of cell death by apoptosis, a massively occurring physiological process required for tissue homeostasis. The clearance of apoptotic cells is a highly efficient process and is suggested to result in an anti-inflammatory or tolerizing effect. However, in many clinical conditions, including rheumatic diseases, the process of clearing dying and dead cells is overwhelmed by either too much cell death or too little clearance. Complement plays an important role in the recognition and efficient phagocytosis of apoptotic and necrotic cells [19]. A complement system malfunctioning as a consequence of genetic, acquired or therapy-induced deficiencies can thus impact the clearance efficiency and cause accumulation of autoantigens, which are released from uncleared dead or dying cells. Several complement proteins have the capacity to bind to dead cells, prominently C1q, but also

MBL, ficolins, collectins, and properdin [20-25]. These complement proteins bind a wide variety of exposed ligands on dead cells [26]. Interestingly, some cells downregulate the expression of membrane bound complement inhibitors, CD46, CD55 and CD59 during apoptosis, potentially leaving the cells vulnerable for complement attack [27]. However, while cells decrease the expression of membrane bound complement inhibitors, they gain the capacity to bind the fluid phase complement inhibitors factor H and C4BP [28]. A wide set of ligands can mediate such binding, but factor H and C4BP can also directly bind to exposed DNA [29].

Complement in rheumatic disease

The complement system is an important factor in host defense, but defects leading to uncontrolled complement activation can cause a variety of clinical conditions. Here, we focus on three major rheumatic diseases in which complement plays an important role and for which complement inhibitory therapy was recently initiated in therapeutic trials: rheumatoid arthritis, systemic lupus erythematosus (SLE) and ANCA-associated vasculitis (AAV). We describe the current knowledge on the influence of complement activation and regulation within these diseases, both endogenous and therapy-induced.

Rheumatoid arthritis & complement

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which affects up to 1% of the population [30, 31]. The main symptoms are located primarily in the small peripheral joints, where chronic inflammation causes damage to cartilage and bone through mechanisms involving both the innate and adaptive parts of the immune system. The lining of the joint, the synovium, is infiltrated by both T and B lymphocytes, as well as macrophages [32, 33]. Later in disease progression, the cartilage and bone in the joint are invaded from the synovium during the formation of a ‘pannus’, causing damage through resorption and breakdown of bone by osteoclasts [34]. The release of proteolytic enzymes like cathepsins and metalloproteinases also lead to the breakdown of matrix proteins in cartilage and bone [35].

Apart from this cellular immune involvement, humoral immunity also plays a major role in RA. Autoantibodies are estimated to be present in 50% to 80% of RA patients [36]. Prominent among these autoantibodies are rheumatoid factor, anti-collagen type II antibodies and antibodies against post-translationally modified proteins, such as anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) [37-40]. The autoantibodies present in RA patients often target antigens in cartilage and the

synovium, contributing to the formation of immune complexes. Crucially, these immune complexes can activate complement, which gives rise to chronic destruction of the joint, via the initiation of innate as well as adaptive immune responses. Indeed, it was shown that ACPA can activate complement via both the CP and AP [41].

A possible role for complement in RA was initially concluded from studies that found activated or cleaved complement components in the synovial fluid [42-47]. Later, signs of complement activation, such as C1q-C4 complexes, were also identified in the circulation of RA patients [48]. The activating role of complement in RA is illustrated in collagen induced arthritis (CIA) models, where mice with a compromised complement system are less affected by the disease [49-51]. Currently, it is still not completely known which ligands are triggering this complement activation.

Several animal models for arthritis are regularly used to study pathogenic mechanisms. Several studies in animal models of arthritis, such as the collagen antibody-induced arthritis (CAIA) model, revealed that exclusively the alternative pathway is essential for disease development [52-54]. Although the classical pathway does contribute to disease, it was not essential in these models. The large role of the alternative pathway in RA makes research into its regulators very interesting. Factor H and its variant, factor H-like protein 1 (FHL-1), may have a protective role against complement damage in the synovium [55]. Both proteins are expressed by synovial fibroblasts from RA patients and their production was increased after stimulation with either IFN-γ or dexamethasone [56]. Intervention with recombinant complement receptor 2 linked factor H (CR2-FH) in a CAIA model showed decreased disease activity and C3 deposition in the synovium [57]. Another study in the same model showed that complement activation was increased when the tissue binding domain of factor H was blocked, providing more evidence that factor H plays a vital role against arthritis in this mouse model [58]. Additionally, autoantibodies against factor H were found with higher frequency in RA patients compared to healthy controls [59]. Single nucleotide polymorphisms (SNPs) of factor H known for their association to macular degeneration, were however not found to correlate with development of RA [60].

RA joints may contain apoptotic and necrotic cells due to prior damage, which can trigger complement activation [20]. Furthermore, complement can be activated by proteins originating from the extracellular matrix (ECM) of damaged cartilage in the joint, like osteoadherin, fibromodulin, aggrecan and cartilage oligomeric matrix protein (COMP) [61-64]. For example, levels of COMP-C3b complexes were found to be elevated in RA patients compared to healthy controls [65]. Several ECM proteins also bind C4BP and

specific ECM proteins with inhibitory properties, such as decorin, have been described as well [61, 66, 67]. Due to complement activation on proteins from the ECM, RA patients often have increased concentrations of complement activation fragments and show signs of higher C4 and C3 consumption in the synovium. Complement activation by immune complexes and other protein triggers is thought to be a mediator of inflammation, attracting effector cells mainly through the anaphylatoxin C5a, again stimulating the inflammatory state in the joint [68]. Another consequence of complement activation in the joint is the direct stimulation of collagenase production in synovial fibroblasts by sublytic levels of the membrane attack complex [69].

Multiple complement proteins, such as C1q and C1-INH, are also synthesized locally in the synovium next to their systemic production, suggesting a local role [70]. Other fluid phase regulators were also investigated: In a CIA model, mice that were given human C4BP showed later disease onset. When C4BP was given only after onset, mice showed reduced disease severity [71]. The lectin pathway regulator MAp44 also plays a role in limiting complement activation in a CAIA model in mice [72]. In this study, adenovirus programmed to express human MAp44 was injected into mice, which resulted in a significant reduction of the disease activity score. Deficiency of another fluid phase regulator, carboxypeptidase B, was found to aggravate arthritis in a mouse model [73]. Moreover, an allele encoding a carboxypeptidase B variant with longer half-life was linked to a decreased risk of developing severe disease in RA patients [73].

The membrane bound complement regulator CD55, also known as decay-accelerating factor (DAF), is an important factor that protects cells from complement attack by dissociating C3 and C5 convertases. Expression of CD55 on fibroblast-like synoviocytes is actually found to be high compared to leukocytes and endothelial cells [74]. Next to a role in complement, CD55 can also act as a ligand for CD97. CD97 is expressed on a variety of cells including infiltrating macrophages and T cells, and may thereby contribute to continued inflammation in the joint via other mechanisms as well [75, 76]. These results indicate that CD55 both regulates complement activation in the joint and contributes to immune cell infiltration and inflammation.

Since the animal models suggest a crucial role of complement in the disease process of RA, intervention studies with complement inhibitors have also been performed in RA. Surprisingly, despite the observed complement activation in human joints and the compelling evidence in mice, there was no benefit in RA of blocking the C5a – C5a receptor interaction [77]. Studies using eculizumab to block cleavage of C5 were not

sufficiently beneficial for further trials either. Consequently, there is no obvious major role for complement therapeutics reported so far in RA, highlighting the disparity between animal models and patients.

Systemic lupus erythematosus & complement

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease that affects between 0.01% and 0.1% of the population. SLE mainly affects women around the child bearing age. SLE is a systemic disease where many organs are affected, either simultaneously or consecutively. Organs affected in this disease include, but are not limited to, the skin, joints, kidney and brain. Especially the involvement of the kidney, called lupus nephritis, is a major contributor to morbidity. The recent focus on brain involvement in lupus also increased the awareness of neuropsychiatric complications [78]. SLE is characterized by the generation of a large variety of autoantibodies (reviewed in [79]). The hallmark autoantibodies in SLE are generated against moieties that are normally not exposed outside the cell, such as double stranded DNA and nuclear proteins. The autoantibodies characteristic of SLE form immune complexes with these nuclear components when they are released from dead cells. Most symptoms of SLE are thought to result from immune complexes formed by these autoantibodies, which deposit in tissue and subsequently cause an inflammatory response by binding complement and Fc receptors. Circulating immune complexes activate complement too, although clusterin and vitronectin were found to offer some protection by their presence on circulating immune complexes containing precursors to MAC [80]. First evidence of complement involvement in SLE came from findings of complement deposition in affected organs, followed later by observations of decreased levels of complement factors and increased levels of complement activation fragments were found [81-83].

The role of complement in SLE is two-fold. On the one hand, complement activation is important for inflammation and tissue damage in most patients. On the other hand, rare genetic deficiencies of several complement components predispose for development of SLE [84]. Deficiencies in the classical pathway give a high risk of developing SLE, with deficiency of C1q causing the highest risk [85]. Several of such C1q deficient patients have been described [86-88]. Noticeably, there is a wide heterogeneity in the clinical presentation and severity of disease in these patients [89, 90]. Especially in C1q deficient SLE patients, a high proportion of patients experience neurological problems, which may be related to a role for C1q in shaping or maintaining nerve interactions [86, 91]. Why C1q deficient patients have such a high risk of developing SLE is currently not certain. Several mechanisms have been suggested, including the reduced clearance of

apoptotic cells and the reduced suppression of T cell activity [92, 93]. While apoptotic cells are generally not detected in vivo because of their efficient clearance, they were detected in C1q deficient patients, suggesting slower clearance. C1q binds the surface of apoptotic cells and enhances phagocytosis, as discussed in the section Complement activation and regulation. For a strong autoantibody response, T cell activity is required. The reported function of C1q as an inhibitor of T cells responses could fit with the notion that in C1q deficiency T cell help is provided to autoreactive B cells [93]. However, both proposed mechanisms may be insufficient to fully explain the risk to develop SLE and other mechanisms may contribute as well.

The lectin pathway has also been studied in detail in SLE, revealing that the levels of several lectin pathway proteins correlate with the (decreased) levels of C3, which suggests consumption because of activation [94, 95]. Meta-analyses on mutations associated with MBL deficiency indicated an modestly increased risk for SLE in MBL deficient individuals [96, 97]. Furthermore, animal models of SLE suggest activation of the lectin pathway during the course of disease [98]. The serum levels of lectin pathway inhibitors Map44 was reported to be slightly higher and MAp19 to be slightly lower in SLE as compared to healthy controls [95].

Autoantibodies have been described for nearly all complement proteins [99]. In the context of SLE, especially anti-C1q, anti-MBL and anti-ficolin-3 are important [100-102]. Autoantibodies against C1q are associated with lupus nephritis in SLE patients, although anti-C1q antibodies are also present in a small part of the healthy population without signs of renal problems [103, 104]. Mouse studies have shown that the contribution of anti-C1q autoantibodies to lupus nephritis is dependent on the prior presence of C1q binding immune complexes in the glomeruli [105, 106]. For anti-ficolin antibodies an association with lupus nephritis was also reported, while for the anti-MBL such association was not observed [101, 102]. The functional consequences of anti-MBL autoantibodies to the pathogenesis is currently unclear [101, 107-109]. Antibodies against soluble regulators were also found. For example, anti-C1-INH autoantibodies were found to be increased in SLE patients and were associated with high disease activity scores [110]. Factor H antibodies were also found in SLE patients, but not at a significantly higher rate than in healthy controls [59].

An important player in the clearance of immune complexes, complement receptor 1 (CR1), is found on several types of myeloid cells, including monocytes, dendritic cells and erythrocytes. CR1 binds targets opsonized by C4b and C3b, and is involved in the transport and uptake of immune complexes. Erythrocytes of SLE patients were found to have decreased levels of

CR1, while stimulation of erythropoiesis in lupus patients with anemia resulted in improved CR1 function [111, 112]. As a result of reduced CR1, these patients cannot clear new immune complexes as effectively and their erythrocytes are more vulnerable to complement attack, because they have lower complement inhibitory activity of CR1. This may result in a vicious circle that conserves a disease state that is hard to treat.

Membrane-bound complement regulatory proteins are important to protect cells from complement activation. Decreased levels of CD55 and CD59 were found on lymphocytes in SLE patients, which may contribute to lymphopenia in these individuals [113, 114]. In serum of patients with active SLE, increased levels of soluble CD46 were found, compared to healthy controls or patients with other autoimmune diseases [115]. The higher levels soluble CD46 may be explained by increased CD46 shedding by T helper 1 cells, which is related to hyperactivity and defective contraction in these cells [116].

Soluble regulators are naturally of great importance as well, and their disruption may cause serious complement damage. Factor I levels in SLE patients were found to be negatively correlated with disease activity score [117]. Some SLE patients with lupus nephritis were described to have dysfunctional factor H. Purified factor H from 3 out of 4 patients induced less phagocytosis of late apoptotic cells and protection against lysis of erythrocytes was decreased in 2 of the patients [118]. Factor H, as well as C4BP, is also important in the protection of apoptotic cells against uncontrolled complement activation and lysis [28]. A large case-control study on factor H and factor H-related genes concluded that a CFHR3-1 deletion was associated with SLE and might contribute to development of the disease [119]. The impact of factor H is underlined in an animal study, where factor H deficient MRL-lpr mice showed accelerated development of lupus nephritis compared to factor H sufficient mice [120].

In view of the broad involvement of complement in SLE, it seems evident that therapeutic targeting of complement in SLE patients could be beneficial. However, complement targeted therapy has so far only been used sporadically in SLE. Genetic deficiencies of classical pathway components were successfully overcome by repeated plasma infusions, which resulted in disappearance of SLE symptoms for weeks after infusion [88, 121]. Transplantation of hematopoietic stem cells has also been tested in C1q deficient individuals; clinical results were good for two patients, while a third did not survive [87, 122]. Eculizumab has been used in a few patients where excessive complement activation contributes to disease, with good results [123, 124]. Another recent study investigated the treatment of SLE by mesenchymal stem cell (MSC) transplantation, obtaining either a major or partial clinical response in the majority of patients [125].

This clinical effect of MSC transplantation was reportedly due to increased factor H production [126]. However, more studies will be needed before complement-targeting therapy could be applied on a large scale in SLE patients.

ANCA-associated vasculitis & complement

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a disease that covers multiple clinical conditions including granulomatosis with polyangiitis (GPA), eosinophilic GPA and microscopic polyangiitis. These diseases have in common that the vast majority of patients (90%) are positive for ANCA [127]. Two different neutrophil proteins are recognized as targets for ANCA: proteinase 3 (PR3) and myeloperoxidase (MPO) [128, 129]. In a resting state, these targets are only found intracellularly, but priming through complement activation fragment or cytokine-mediated stimulation causes the ANCA antigens to also be expressed on the cell surface. The exposed ANCA antigens can now be recognized by the antibodies. Neutrophils are activated by ANCA binding, resulting in degranulation and migration into the wall of the blood vessel, thereby contributing to vasculitis [130]. The full pathogenesis of AAV has recently been reviewed [131]. Renal involvement in AAV is characterized by pauci-immune glomerulonephritis [132]. The histological lesions in the kidney are characterized by crescents and fibrinoid necrosis in the absence of a pronounced presence of immune complexes or complement [127]. Because of this near absence of complement activation staining in the kidney and the absence of hypocomplementemia, no role for complement was anticipated at first. This view changed when a role for complement in AAV was shown in mouse models: the development of ANCA-induced glomerulonephritis was shown to be dependent on alternative pathway activation and C5a signaling [133].

Next to the neutrophil activation by C5a, complement also plays an amplifying role after the initial neutrophil activation. Binding of ANCA to its antigen on the neutrophil can give rise to a range of actions, such as degranulation, neutrophil extracellular trap (NET) formation and respiratory bursts. Neutrophil activation can also cause novel complement activation. One study found that complement in human serum is activated upon addition of supernatant from ANCA-stimulated neutrophils, but not by supernatant of unstimulated neutrophils, measured at the level of C5a [134]. Consequently, there is a local increase in leukocyte infiltration, neutrophil activation and tissue damage, again continuing the inflammatory state [135]. The secondary complement activation seems to be mediated through the alternative pathway. In mouse models of vasculitis induced by MPO-ANCA, C4 deficient mice (lacking classical and lection pathway activation) were susceptible to development of glomerulonephritis and vasculitis, while animals

deficient for alternative pathway activation were protected from the disease [133]. How this alternative pathway activation is triggered remains unclear, but activation of the alternative pathway by activated platelets was recently suggested [136].

As the main regulator of the alternative pathway, factor H could be an important player in limiting secondary complement activation. Indeed, plasma factor H levels were significantly lower in patients with active AAV as compared to AAV patients in remission and healthy controls [137]. Further research from the same group showed that factor H from AAV patients is often less active in binding and regulating C3b and in guarding self-cells against complement damage [138]. Neutrophils activated by ANCA can release myeloperoxidase, which in turn can bind factor H and inhibit the interaction between factor H and C3b [139]. Interestingly, normal factor H binds to neutrophils where it can inhibit ANCA-induced activation and degranulation. In a coculture system of neutrophils and human glomerular endothelial cells, addition of factor H increased migration of neutrophils towards the endothelial cells and decreased activation of and damage to endothelial cells [140]. In the same study, it was found that factor H from AAV patients was dysfunctional in the binding to neutrophils, indicating a role for factor H in the pathogenesis of AAV.

Despite involvement of multiple complement factors in AAV, C5 remains a key player [134, 141]. Recently, blocking of the C5a receptor (C5aR) was shown to protect mice against ANCA induced glomerulonephritis [142]. In this study, C5aR deficiency ameliorated the effect of induced glomerulonephritis, while animals with human C5aR showed similar disease as wildtype mice. Importantly, the human C5aR inhibitor CCX168 was used to block human C5aR in mice, resulting in drastically less necrosis and crescents in the glomeruli and lower neutrophil infiltration in the glomeruli [142]. Subsequently, a Phase 1 clinical trial was started, which showed that CCX168 (now also called avacopan) was tolerated in humans without major issues [143]. Further studies where CCX168 partially or fully replaced the use of prednisone in AAV patients displayed effectiveness at least similar to the current standard treatment for these patients in terms of nephritis inhibition [144]. These promising trails could open the door to novel complement-directed therapy in the field of rheumatic diseases where, as for AAV, initially a role for complement was not anticipated.

Discussion

The complement system is important in many physiological processes, but can also be a mediator of disease. The involvement of complement in rheumatic diseases is often deduced from the presence of complement deposition in affected tissue and/or increased levels of complement activation fragments. This does not necessarily mean

that complement plays a causal role in each of the rheumatic diseases discussed here. Present literature is largely focused on the activating ligands and complement factors as opposed to complement regulators. However, it is not always clear whether aberrant complement activity is the result of excess activation or reduced regulation. Especially in RA and SLE, a reasonable number of complement components have been related to the disease in one way or another. Nevertheless, for many it remains unknown whether these associations could be used as predictive biomarkers or therapeutic targets. This is a theme that could definitely benefit from deeper investigation in the future. In AAV, the key complement component that contributes to disease is C5, acting mainly through C5a signaling, with a promising C5aR inhibitor currently being studied in clinical trial. Such complement targeting therapy seems less likely for RA in the near future, since several other treatment options are already available. The balance between activation and regulation of complement seems particularly important in SLE, where reduced complement activation can impede clearance of potential sources of autoantigens like apoptotic cells, while increased activation on deposited immune complexes contributes to tissue damage. Although a small number of SLE patients has been treated with eculizumab with good results, a broader investigation is needed before this therapy could be applied on a larger scale. A key contribution in complement-targeting therapy could be made by natural complement regulators or mutant variants thereof. Another interesting feature of future complement therapy may be locally targeted regulation instead of systemic inhibition. This gives the advantage that the complement system is still capable of functioning normally in nonaffected parts of the body. With the advent of the first complement therapeutics, and the probable arrival of more in the future, there seem to be multiple possible applications in the treatment of rheumatic diseases. The three rheumatic diseases highlighted in this review all display clear evidence of complement activation, also the animal models for these diseases indicate an active role for complement in the disease process. Strikingly at this moment therapeutic complement inhibition appears only to be effective in AAV and not in RA or SLE. Clearly there is a need for a more thorough understanding of the contribution of complement activation and complement regulation to the disease processes in rheumatic diseases.

Acknowledgements

The authors wish to acknowledge the financial support from the Leiden University Medical Center profile area Infection, Immunity and Tolerance. L.A.T. has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 724517).

References

1. D. Ricklin, G. Hajishengallis, K. Yang, J. D. Lambris, Complement: A key system for immune surveillance and homeostasis. *Nature immunology* **11**, 785 (2010).
2. F. J. Beurskens, R. A. van Schaarenburg, L. A. Trouw, C1q, antibodies and anti-c1q autoantibodies. *Molecular immunology* **68**, 6-13 (2015).
3. D. Ugurlar, S. C. Howes, B.-J. de Kreuk, R. I. Koning, R. N. de Jong, F. J. Beurskens, J. Schuurman, A. J. Koster, T. H. Sharp, P. W. Parren, Structures of c1-igg1 provide insights into how danger pattern recognition activates complement. *Science* **359**, 794-797 (2018).
4. P. Garred, N. Genster, K. Pilely, R. Bayarri-Olmos, A. Rosbjerg, Y. J. Ma, M. O. Skjoedt, A journey through the lectin pathway of complement—mbl and beyond. *Immunological reviews* **274**, 74-97 (2016).
5. T. Fujita, Evolution of the lectin–complement pathway and its role in innate immunity. *Nature Reviews Immunology* **2**, 346 (2002).
6. R. Pouw, D. Vredevoogd, T. Kuijpers, D. Wouters, Of mice and men: The factor h protein family and complement regulation. *Molecular immunology* **67**, 12-20 (2015).
7. M. Harboe, T. E. Mollnes, The alternative complement pathway revisited. *Journal of cellular and molecular medicine* **12**, 1074-1084 (2008).
8. T. H. Sharp, A. J. Koster, P. Gros, Heterogeneous mac initiator and pore structures in a lipid bilayer by phase-plate cryo-electron tomography. *Cell reports* **15**, 1-8 (2016).
9. R. Lubbers, M. F. van Essen, C. van Kooten, L. A. Trouw, Production of complement components by cells of the immune system. *Clin Exp Immunol* **188**, 183-194 (2017).
10. R. T. White, D. Damm, N. Hancock, B. Rosen, B. Lowell, P. Usher, J. Flier, B. Spiegelman, Human adipsin is identical to complement factor d and is expressed at high levels in adipose tissue. *Journal of Biological Chemistry* **267**, 9210-9213 (1992).
11. H. Hartung, U. Hadding (1983) Synthesis of complement by macrophages and modulation of their functions through complement activation. in *Springer seminars in immunopathology* (Springer), pp 283-326.
12. G. Castellano, L. A. Trouw, N. Fiore, M. R. Daha, F. P. Schena, C. van Kooten, Infiltrating dendritic cells contribute to local synthesis of c1q in murine and human lupus nephritis. *Molecular immunology* **47**, 2129-2137 (2010).
13. R. A. van Schaarenburg, J. Suurmond, K. L. Habets, M. C. Brouwer, D. Wouters, F. A. Kurreeman, T. W. Huizinga, R. E. Toes, L. A. Trouw, The production and secretion of complement component c1q by human mast cells. *Molecular immunology* **78**, 164-170 (2016).
14. A. Sjöberg, L. Trouw, A. Blom, Complement activation and inhibition: A delicate balance. *Trends in immunology* **30**, 83-90 (2009).
15. D. Ricklin, E. S. Reis, J. D. Lambris, Complement in disease: A defence system turning offensive. *Nature Reviews Nephrology* **12**, 383 (2016).
16. M. Cicardi, L. Zingale, A. Zanichelli, E. Pappalardo, B. Cicardi (2005) C1 inhibitor: Molecular and clinical aspects. in *Springer seminars in immunopathology* (Springer), pp 286-298.
17. S. E. Degn, A. G. Hansen, R. Steffensen, C. Jacobsen, J. C. Jensenius, S. Thiel, Map44, a human protein associated with pattern recognition molecules of the complement system and regulating the lectin pathway of complement activation. *The Journal of Immunology* **183**, 7371-7378 (2009).

18. D. Ermert, A. M. Blom, C4b-binding protein: The good, the bad and the deadly. Novel functions of an old friend. *Immunology letters* **169**, 82-92 (2016).
19. L. Trouw, A. Blom, P. Gasque, Role of complement and complement regulators in the removal of apoptotic cells. *Molecular immunology* **45**, 1199-1207 (2008).
20. A. J. Nauta, L. A. Trouw, M. R. Daha, O. Tijsma, R. Nieuwland, W. J. Schwaeble, A. R. Gingras, A. Mantovani, E. C. Hack, A. Roos, Direct binding of c1q to apoptotic cells and cell blebs induces complement activation. *European journal of immunology* **32**, 1726-1736 (2002).
21. A. J. Nauta, N. Raaschou-Jensen, A. Roos, M. R. Daha, H. O. Madsen, M. C. Borrias-Essers, L. P. Ryder, C. Koch, P. Garred, Mannose-binding lectin engagement with late apoptotic and necrotic cells. *European journal of immunology* **33**, 2853-2863 (2003).
22. M. Kuraya, Z. Ming, X. Liu, M. Matsushita, T. Fujita, Specific binding of l-ficolin and h-ficolin to apoptotic cells leads to complement activation. *Immunobiology* **209**, 689-697 (2005).
23. L. M. Stuart, P. M. Henson, R. W. Vandivier, "Collectins: Opsonins for apoptotic cells and regulators of inflammation" in Apoptosis and its relevance to autoimmunity. (Karger Publishers, 2006), vol. 9, pp. 143-161.
24. W. Xu, S. P. Berger, L. A. Trouw, H. C. de Boer, N. Schlagwein, C. Mutsaers, M. R. Daha, C. van Kooten, Properdin binds to late apoptotic and necrotic cells independently of c3b and regulates alternative pathway complement activation. *The Journal of Immunology* **180**, 7613-7621 (2008).
25. C. Kemper, L. M. Mitchell, L. Zhang, D. E. Hourcade, The complement protein properdin binds apoptotic t cells and promotes complement activation and phagocytosis. *Proceedings of the National Academy of Sciences* **105**, 9023-9028 (2008).
26. M. Martin, A. M. Blom, Complement in removal of the dead–balancing inflammation. *Immunological reviews* **274**, 218-232 (2016).
27. D. S. Cole, T. R. Hughes, P. Gasque, B. P. Morgan, Complement regulator loss on apoptotic neuronal cells causes increased complement activation and promotes both phagocytosis and cell lysis. *Molecular immunology* **43**, 1953-1964 (2006).
28. L. A. Trouw, A. A. Bengtsson, K. A. Gelderman, B. Dahlbäck, G. Sturfelt, A. M. Blom, C4b-binding protein and factor h compensate for the loss of membrane-bound complement inhibitors to protect apoptotic cells against excessive complement attack. *Journal of Biological Chemistry* **282**, 28540-28548 (2007).
29. L. A. Trouw, S. C. Nilsson, I. Gonçalves, G. Landberg, A. M. Blom, C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. *Journal of Experimental Medicine* **201**, 1937-1948 (2005).
30. A. J. Silman, J. E. Pearson, Epidemiology and genetics of rheumatoid arthritis. *Arthritis research & therapy* **4**, S265 (2002).
31. Y. Alamanos, P. V. Voulgari, A. A. Drosos (2006) Incidence and prevalence of rheumatoid arthritis, based on the 1987 american college of rheumatology criteria: A systematic review. in *Seminars in arthritis and rheumatism* (Elsevier), pp 182-188.
32. C. L. Young, T. C. Adamson III, J. H. Vaughan, R. I. Fox, Immunohistologic characterization of synovial membrane lymphocytes in rheumatoid arthritis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **27**, 32-39 (1984).

33. P. P. Tak, T. J. Smeets, M. R. Daha, P. M. Kluin, K. A. Meijers, R. Brand, A. E. Meinders, F. C. Breedveld, Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. *Arthritis & Rheumatism* **40**, 217-225 (1997).

34. S. B. Cohen, R. K. Dore, N. E. Lane, P. A. Ory, C. G. Peterfy, J. T. Sharp, D. Van Der Heijde, L. Zhou, W. Tsuji, R. Newmark, Denosumab treatment effects on structural damage, bone mineral density, and bone turnover in rheumatoid arthritis: A twelve-month, multicenter, randomized, double-blind, placebo-controlled, phase ii clinical trial. *Arthritis & Rheumatism* **58**, 1299-1309 (2008).

35. M. W. Lark, E. K. Bayne, J. Flanagan, C. F. Harper, L. A. Hoerrner, N. I. Hutchinson, I. I. Singer, S. A. Donatelli, J. R. Weidner, H. R. Williams, Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *The Journal of clinical investigation* **100**, 93-106 (1997).

36. D. L. Scott, F. Wolfe, T. W. Huizinga, Rheumatoid arthritis. *Lancet* **376**, 1094-1108 (2010).

37. E. M. Tan, J. S. Smolen, Historical observations contributing insights on etiopathogenesis of rheumatoid arthritis and role of rheumatoid factor. *J Exp Med* **213**, 1937-1950 (2016).

38. L. A. Trouw, T. Rispens, R. E. M. Toes, Beyond citrullination: Other post-translational protein modifications in rheumatoid arthritis. *Nat Rev Rheumatol* **13**, 331-339 (2017).

39. L. Klareskog, J. Ronnelid, K. Lundberg, L. Padyukov, L. Alfredsson, Immunity to citrullinated proteins in rheumatoid arthritis. *Annu Rev Immunol* **26**, 651-675 (2008).

40. J. Shi, R. Knevel, P. Suwannalai, M. P. van der Linden, G. M. Janssen, P. A. van Veelen, N. E. Levarht, A. H. van der Helm-van Mil, A. Cerami, T. W. Huizinga, R. E. Toes, L. A. Trouw, Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A* **108**, 17372-17377 (2011).

41. L. A. Trouw, E. M. Haisma, E. W. Levarht, D. van der Woude, A. Ioan-Facsinay, M. R. Daha, T. W. Huizinga, R. E. Toes, Anti-cyclic citrullinated peptide antibodies from rheumatoid arthritis patients activate complement via both the classical and alternative pathways. *Arthritis Rheum* **60**, 1923-1931 (2009).

42. J. P. Brodeur, S. Ruddy, L. B. Schwartz, G. Moxley, Synovial fluid levels of complement sc5b-9 and fragment bb are elevated in patients with rheumatoid arthritis. *Arthritis Rheum* **34**, 1531-1537 (1991).

43. A. J. Swaak, A. Van Rooyen, O. Planten, H. Han, O. Hattink, E. Hack, An analysis of the levels of complement components in the synovial fluid in rheumatic diseases. *Clin Rheumatol* **6**, 350-357 (1987).

44. G. Moxley, S. Ruddy, Elevated c3 anaphylatoxin levels in synovial fluids from patients with rheumatoid arthritis. *Arthritis Rheum* **28**, 1089-1095 (1985).

45. T. E. Mollnes, A. Paus, Complement activation in synovial fluid and tissue from patients with juvenile rheumatoid arthritis. *Arthritis Rheum* **29**, 1359-1364 (1986).

46. R. D. Inman, P. C. Harpel, C1 inactivator-c1s complexes in inflammatory joint disease. *Clin Exp Immunol* **53**, 521-528 (1983).

47. M. Shingu, Y. Watanabe, K. Tomooka, K. Yoshioka, E. Ohtsuka, M. Nobunaga, Complement degradation products in rheumatoid arthritis synovial fluid. *Br J Rheumatol* **33**, 299-300 (1994).

48. D. Wouters, A. E. Voskuyl, E. T. Molenaar, B. A. Dijkmans, C. E. Hack, Evaluation of classical complement pathway activation in rheumatoid arthritis: Measurement of c1q-c4 complexes as novel activation products. *Arthritis Rheum* **54**, 1143-1150 (2006).

49. N. K. Banda, D. Kraus, A. Vondracek, L. H. Huynh, A. Bendele, V. M. Holers, W. P. Arend, Mechanisms of effects of complement inhibition in murine collagen-induced arthritis. *Arthritis Rheum* **46**, 3065-3075 (2002).

50. M. A. Hietala, I. M. Jonsson, A. Tarkowski, S. Kleinau, M. Pekna, Complement deficiency ameliorates collagen-induced arthritis in mice. *J Immunol* **169**, 454-459 (2002).

51. M. A. Hietala, K. S. Nandakumar, L. Persson, S. Fahlen, R. Holmdahl, M. Pekna, Complement activation by both classical and alternative pathways is critical for the effector phase of arthritis. *Eur J Immunol* **34**, 1208-1216 (2004).

52. N. K. Banda, K. Takahashi, A. K. Wood, V. M. Holers, W. P. Arend, Pathogenic complement activation in collagen antibody-induced arthritis in mice requires amplification by the alternative pathway. *J Immunol* **179**, 4101-4109 (2007).

53. N. K. Banda, J. M. Thurman, D. Kraus, A. Wood, M. C. Carroll, W. P. Arend, V. M. Holers, Alternative complement pathway activation is essential for inflammation and joint destruction in the passive transfer model of collagen-induced arthritis. *J Immunol* **177**, 1904-1912 (2006).

54. H. Ji, K. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, A. Ezekowitz, M. C. Carroll, M. Brenner, R. Weissleder, J. S. Verbeek, V. Duchatelle, C. Degott, C. Benoist, D. Mathis, Arthritis critically dependent on innate immune system players. *Immunity* **16**, 157-168 (2002).

55. J. Ferluga, L. Kouser, V. Murugaiah, R. B. Sim, U. Kishore, Potential influences of complement factor h in autoimmune inflammatory and thrombotic disorders. *Mol Immunol* **84**, 84-106 (2017).

56. M. A. Friese, T. Manuelian, S. Junnikkala, J. Hellwage, S. Meri, H. H. Peter, D. L. Gordon, H. Eibel, P. F. Zipfel, Release of endogenous anti-inflammatory complement regulators fhl-1 and factor h protects synovial fibroblasts during rheumatoid arthritis. *Clin Exp Immunol* **132**, 485-495 (2003).

57. N. K. Banda, B. Levitt, M. J. Glogowska, J. M. Thurman, K. Takahashi, G. L. Stahl, S. Tomlinson, W. P. Arend, V. M. Holers, Targeted inhibition of the complement alternative pathway with complement receptor 2 and factor h attenuates collagen antibody-induced arthritis in mice. *J Immunol* **183**, 5928-5937 (2009).

58. N. K. Banda, G. Mehta, V. P. Ferreira, C. Cortes, M. C. Pickering, M. K. Pangburn, W. P. Arend, V. M. Holers, Essential role of surface-bound complement factor h in controlling immune complex-induced arthritis. *J Immunol* **190**, 3560-3569 (2013).

59. A. Foltyn Zadura, P. F. Zipfel, M. I. Bokarewa, G. Sturfelt, A. Jonsen, S. C. Nilsson, A. Hillarp, T. Saxne, L. A. Trouw, A. M. Blom, Factor h autoantibodies and deletion of complement factor h-related protein-1 in rheumatic diseases in comparison to atypical hemolytic uremic syndrome. *Arthritis Res Ther* **14**, R185 (2012).

60. L. A. Trouw, S. Bohringer, N. A. Daha, E. A. Stahl, S. Raychaudhuri, F. A. Kurreeman, G. Stoeken-Rijsbergen, J. J. Houwing-Duistermaat, T. W. Huizinga, R. E. Toes, The major risk alleles of age-related macular degeneration (amd) in cfh do not play a major role in rheumatoid arthritis (ra). *Clin Exp Immunol* **166**, 333-337 (2011).

61. A. P. Sjoberg, G. A. Manderson, M. Morgelin, A. J. Day, D. Heinegard, A. M. Blom, Short leucine-rich glycoproteins of the extracellular matrix display diverse patterns of complement interaction and activation. *Mol Immunol* **46**, 830-839 (2009).

62. A. Sjoberg, P. Onnerfjord, M. Morgelin, D. Heinegard, A. M. Blom, The extracellular matrix and inflammation: Fibromodulin activates the classical pathway of complement by directly binding c1q. *J Biol Chem* **280**, 32301-32308 (2005).

63. C. Melin Furst, M. Morgelin, K. Vadstrup, D. Heinegard, A. Aspberg, A. M. Blom, The c-type lectin of the aggrecan g3 domain activates complement. *PLoS One* **8**, e61407 (2013).

64. K. E. Happonen, T. Saxne, A. Aspberg, M. Morgelin, D. Heinegard, A. M. Blom, Regulation of complement by cartilage oligomeric matrix protein allows for a novel molecular diagnostic principle in rheumatoid arthritis. *Arthritis Rheum* **62**, 3574-3583 (2010).

65. K. E. Happonen, T. Saxne, P. Geborek, M. Andersson, A. A. Bengtsson, R. Hesselstrand, D. Heinegard, A. M. Blom, Serum comp-c3b complexes in rheumatic diseases and relation to anti-tnf-alpha treatment. *Arthritis Res Ther* **14**, R15 (2012).

66. K. E. Happonen, A. P. Sjoberg, M. Morgelin, D. Heinegard, A. M. Blom, Complement inhibitor c4b-binding protein interacts directly with small glycoproteins of the extracellular matrix. *J Immunol* **182**, 1518-1525 (2009).

67. T. W. Groeneveld, M. Oroszlan, R. T. Owens, M. C. Faber-Krol, A. C. Bakker, G. J. Arlaud, D. J. McQuillan, U. Kishore, M. R. Daha, A. Roos, Interactions of the extracellular matrix proteoglycans decorin and biglycan with c1q and collectins. *J Immunol* **175**, 4715-4723 (2005).

68. M. Okroj, D. Heinegard, R. Holmdahl, A. M. Blom, Rheumatoid arthritis and the complement system. *Ann Med* **39**, 517-530 (2007).

69. B. Jahn, J. Von Kempis, K. L. Kramer, S. Filsinger, G. M. Hansch, Interaction of the terminal complement components c5b-9 with synovial fibroblasts: Binding to the membrane surface leads to increased levels in collagenase-specific mrna. *Immunology* **78**, 329-334 (1993).

70. S. Breitner, S. Storkel, W. Reichel, M. Loos, Complement components c1q, c1r/c1s, and c1inh in rheumatoid arthritis. Correlation of in situ hybridization and northern blot results with function and protein concentration in synovium and primary cell cultures. *Arthritis Rheum* **38**, 492-498 (1995).

71. A. M. Blom, K. S. Nandakumar, R. Holmdahl, C4b-binding protein (c4bp) inhibits development of experimental arthritis in mice. *Ann Rheum Dis* **68**, 136-142 (2009).

72. N. K. Banda, G. Mehta, T. R. Kjaer, M. Takahashi, J. Schaack, T. E. Morrison, S. Thiel, W. P. Arend, V. M. Holers, Essential role for the lectin pathway in collagen antibody-induced arthritis revealed through use of adenovirus programming complement inhibitor map44 expression. *J Immunol* **193**, 2455-2468 (2014).

73. J. J. Song, I. Hwang, K. H. Cho, M. A. Garcia, A. J. Kim, T. H. Wang, T. M. Lindstrom, A. T. Lee, T. Nishimura, L. Zhao, J. Morser, M. Nesheim, S. B. Goodman, D. M. Lee, S. L. Bridges, Jr., P. K. Gregersen, L. L. Leung, W. H. Robinson, Plasma carboxypeptidase b downregulates inflammatory responses in autoimmune arthritis. *J Clin Invest* **121**, 3517-3527 (2011).

74. J. Hamann, J. O. Wishaupt, R. A. van Lier, T. J. Smeets, F. C. Breedveld, P. P. Tak, Expression of the activation antigen cd97 and its ligand cd55 in rheumatoid synovial tissue. *Arthritis Rheum* **42**, 650-658 (1999).

75. L. Visser, A. F. de Vos, J. Hamann, M. J. Melief, M. van Meurs, R. A. van Lier, J. D. Laman, R. Q. Hintzen, Expression of the egf-tm7 receptor cd97 and its ligand cd55 (daf) in multiple sclerosis. *J Neuroimmunol* **132**, 156-163 (2002).
76. R. M. Hoek, D. de Launay, E. N. Kop, A. S. Yilmaz-Elis, F. Lin, K. A. Reedquist, J. S. Verbeek, M. E. Medof, P. P. Tak, J. Hamann, Deletion of either cd55 or cd97 ameliorates arthritis in mouse models. *Arthritis Rheum* **62**, 1036-1042 (2010).
77. C. E. Vergunst, D. M. Gerlag, H. Dinant, L. Schulz, M. Vinkenoog, T. J. Smeets, M. E. Sanders, K. A. Reedquist, P. P. Tak, Blocking the receptor for c5a in patients with rheumatoid arthritis does not reduce synovial inflammation. *Rheumatology (Oxford)* **46**, 1773-1778 (2007).
78. C. Magro-Checa, E. J. Zirkzee, T. W. Huizinga, G. M. Steup-Beekman, Management of neuropsychiatric systemic lupus erythematosus: Current approaches and future perspectives. *Drugs* **76**, 459-483 (2016).
79. G. Yaniv, G. Twig, D. B. Shor, A. Furer, Y. Sherer, O. Mozes, O. Komisar, E. Slonimsky, E. Klang, E. Lotan, M. Welt, I. Marai, A. Shina, H. Amital, Y. Shoenfeld, A volcanic explosion of autoantibodies in systemic lupus erythematosus: A diversity of 180 different antibodies found in sle patients. *Autoimmun Rev* **14**, 75-79 (2015).
80. A. K. Chauhan, T. L. Moore, Presence of plasma complement regulatory proteins clusterin (apo j) and vitronectin (s40) on circulating immune complexes (cic). *Clin Exp Immunol* **145**, 398-406 (2006).
81. A. J. Swaak, J. Groenwold, W. Bronsveld, Predictive value of complement profiles and anti-dsdna in systemic lupus erythematosus. *Ann Rheum Dis* **45**, 359-366 (1986).
82. G. Senaldi, V. A. Makinde, D. Vergani, D. A. Isenberg, Correlation of the activation of the fourth component of complement (c4) with disease activity in systemic lupus erythematosus. *Ann Rheum Dis* **47**, 913-917 (1988).
83. S. Manzi, J. E. Rairie, A. B. Carpenter, R. H. Kelly, S. P. Jagarlapudi, S. M. Sereika, T. A. Medsger, Jr., R. Ramsey-Goldman, Sensitivity and specificity of plasma and urine complement split products as indicators of lupus disease activity. *Arthritis Rheum* **39**, 1178-1188 (1996).
84. J. Leffler, A. A. Bengtsson, A. M. Blom, The complement system in systemic lupus erythematosus: An update. *Ann Rheum Dis* **73**, 1601-1606 (2014).
85. G. Sturfelt, L. Truedsson, Complement in the immunopathogenesis of rheumatic disease. *Nat Rev Rheumatol* **8**, 458-468 (2012).
86. R. A. van Schaarenburg, C. Magro-Checa, J. A. Bakker, Y. K. Teng, I. M. Bajema, T. W. Huizinga, G. M. Steup-Beekman, L. A. Trouw, C1q deficiency and neuropsychiatric systemic lupus erythematosus. *Front Immunol* **7**, 647 (2016).
87. P. D. Arkwright, P. Riley, S. M. Hughes, H. Alachkar, R. F. Wynn, Successful cure of c1q deficiency in human subjects treated with hematopoietic stem cell transplantation. *J Allergy Clin Immunol* **133**, 265-267 (2014).
88. P. Mehta, P. J. Norsworthy, A. E. Hall, S. J. Kelly, M. J. Walport, M. Botto, M. C. Pickering, Sle with c1q deficiency treated with fresh frozen plasma: A 10-year experience. *Rheumatology (Oxford)* **49**, 823-824 (2010).
89. R. A. van Schaarenburg, L. Schejbel, L. Truedsson, R. Topaloglu, S. M. Al-Mayouf, A. Riordan, A. Simon, M. Kallel-Sellami, P. D. Arkwright, A. Ahlin, . . . L. A. Trouw, Marked variability in clinical presentation and outcome of patients with c1q immunodeficiency. *J Autoimmun* **62**, 39-44 (2015).

90. M. Stegert, M. Bock, M. Trendelenburg, Clinical presentation of human c1q deficiency: How much of a lupus? *Mol Immunol* **67**, 3-11 (2015).

91. B. Stevens, N. J. Allen, L. E. Vazquez, G. R. Howell, K. S. Christopherson, N. Nouri, K. D. Micheva, A. K. Mehalow, A. D. Huberman, B. Stafford, A. Sher, A. M. Litke, J. D. Lambris, S. J. Smith, S. W. John, B. A. Barres, The classical complement cascade mediates cns synapse elimination. *Cell* **131**, 1164-1178 (2007).

92. M. Botto, C. Dell'Agnola, A. E. Bygrave, E. M. Thompson, H. T. Cook, F. Petry, M. Loos, P. P. Pandolfi, M. J. Walport, Homozygous c1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat Genet* **19**, 56-59 (1998).

93. G. S. Ling, G. Crawford, N. Buang, I. Bartok, K. Tian, N. M. Thielens, I. Bally, J. A. Harker, P. G. Ashton-Rickardt, S. Rutschmann, J. Strid, M. Botto, C1q restrains autoimmunity and viral infection by regulating cd8(+) t cell metabolism. *Science* **360**, 558-563 (2018).

94. A. Troldborg, S. Thiel, L. Jensen, S. Hansen, M. J. Laska, B. Deleuran, J. C. Jensenius, K. Stengaard-Pedersen, Collectin liver 1 and collectin kidney 1 and other complement-associated pattern recognition molecules in systemic lupus erythematosus. *Clin Exp Immunol* **182**, 132-138 (2015).

95. A. Troldborg, S. Thiel, M. Trendelenburg, J. Friebus-Kardash, J. Nehring, R. Steffensen, S. W. K. Hansen, M. J. Laska, B. Deleuran, J. C. Jensenius, A. Voss, K. Stengaard-Pedersen, The lectin pathway of complement activation in patients with systemic lupus erythematosus. *J Rheumatol* **45**, 1136-1144 (2018).

96. W. D. Xu, H. Peng, M. Zhou, M. Zhang, B. Z. Li, H. F. Pan, D. Q. Ye, Association of rantes and mbl gene polymorphisms with systemic lupus erythematosus: A meta-analysis. *Molecular biology reports* **40**, 941-948 (2013).

97. Y. H. Lee, T. Witte, T. Momot, R. E. Schmidt, K. M. Kaufman, J. B. Harley, A. L. Sestak, The mannose-binding lectin gene polymorphisms and systemic lupus erythematosus: Two case-control studies and a meta-analysis. *Arthritis Rheum* **52**, 3966-3974 (2005).

98. L. A. Trouw, M. A. Seelen, J. M. Duijs, S. Wagner, M. Loos, I. M. Bajema, C. van Kooten, A. Roos, M. R. Daha, Activation of the lectin pathway in murine lupus nephritis. *Mol Immunol* **42**, 731-740 (2005).

99. M. A. Dragon-Durey, C. Blanc, M. C. Marinozzi, R. A. van Schaarenburg, L. A. Trouw, Autoantibodies against complement components and functional consequences. *Mol Immunol* **56**, 213-221 (2013).

100. M. Mahler, R. A. van Schaarenburg, L. A. Trouw, Anti-c1q autoantibodies, novel tests, and clinical consequences. *Front Immunol* **4**, 117 (2013).

101. M. A. Seelen, L. A. Trouw, J. W. van der Hoorn, F. C. Fallaux-van den Houten, T. W. Huizinga, M. R. Daha, A. Roos, Autoantibodies against mannose-binding lectin in systemic lupus erythematosus. *Clinical and experimental immunology* **134**, 335-343 (2003).

102. M. Plawecki, E. Lheritier, G. Clavarino, N. Jourde-Chiche, S. Ouili, S. Paul, E. Gout, F. Sarrot-Reynauld, N. Bardin, P. Y. Boelle, L. Chiche, L. Bouillet, N. M. Thielens, J. Y. Cesbron, C. Dumestre-Perard, Association between the presence of autoantibodies targeting ficolin-3 and active nephritis in patients with systemic lupus erythematosus. *PLoS One* **11**, e0160879 (2016).

103. C. Siegert, M. Daha, M. L. Westedt, E. van der Voort, F. Breedveld, Igg autoantibodies against c1q are correlated with nephritis, hypocomplementemia, and dsdna antibodies in systemic lupus erythematosus. *J Rheumatol* **18**, 230-234 (1991).

104. C. E. Siegert, M. R. Daha, A. J. Swaak, E. A. van der Voort, F. C. Breedveld, The relationship between serum titers of autoantibodies to c1q and age in the general population and in patients with systemic lupus erythematosus. *Clin Immunol Immunopathol* **67**, 204-209 (1993).

105. L. A. Trouw, J. M. Duijs, C. van Kooten, M. R. Daha, Immune deposition of c1q and anti-c1q antibodies in the kidney is dependent on the presence of glomerular igg. *Mol Immunol* **40**, 595-602 (2003).

106. L. A. Trouw, T. W. Groeneveld, M. A. Seelen, J. M. Duijs, I. M. Bajema, F. A. Prins, U. Kishore, D. J. Salant, J. S. Verbeek, C. van Kooten, M. R. Daha, Anti-c1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular c1q-containing immune complexes. *J Clin Invest* **114**, 679-688 (2004).

107. M. A. Seelen, E. A. van der Bijl, L. A. Trouw, T. C. Zuiverloon, J. R. Munoz, F. C. Fallaux-van den Houten, N. Schlagwein, M. R. Daha, T. W. Huizinga, A. Roos, A role for mannose-binding lectin dysfunction in generation of autoantibodies in systemic lupus erythematosus. *Rheumatology (Oxford)* **44**, 111-119 (2005).

108. M. Y. Mok, D. L. Jack, C. S. Lau, D. Y. Fong, M. W. Turner, D. A. Isenberg, P. M. Lydyard, Antibodies to mannose binding lectin in patients with systemic lupus erythematosus. *Lupus* **13**, 522-528 (2004).

109. R. Takahashi, A. Tsutsumi, K. Ohtani, D. Goto, I. Matsumoto, S. Ito, N. Wakamiya, T. Sumida, Anti-mannose binding lectin antibodies in sera of japanese patients with systemic lupus erythematosus. *Clin Exp Immunol* **136**, 585-590 (2004).

110. T. Meszaros, G. Fust, H. Farkas, L. Jakab, G. Temesszentandrasi, G. Nagy, E. Kiss, P. Gergely, M. Zeher, Z. Griger, L. Czirjak, R. Hobor, A. Haris, K. Polner, L. Varga, C1-inhibitor autoantibodies in sle. *Lupus* **19**, 634-638 (2010).

111. R. P. Taylor, J. Burge, C. Horgan, D. M. Shasby, The complement-mediated binding of soluble antibody/dsdna immune complexes to human neutrophils. *J Immunol* **130**, 2656-2662 (1983).

112. M. Kavai, Immune complex clearance by complement receptor type 1 in sle. *Autoimmun Rev* **8**, 160-164 (2008).

113. A. P. Alegretti, T. Mucenic, J. Merzoni, G. A. Faulhaber, L. M. Silla, R. M. Xavier, Expression of cd55 and cd59 on peripheral blood cells from systemic lupus erythematosus (sle) patients. *Cell Immunol* **265**, 127-132 (2010).

114. I. Garcia-Valladares, Y. Atisha-Fregoso, Y. Richaud-Patin, J. Jakez-Ocampo, E. Soto-Vega, D. Elias-Lopez, E. Carrillo-Maravilla, J. Cabiedes, A. Ruiz-Arguelles, L. Llorente, Diminished expression of complement regulatory proteins (cd55 and cd59) in lymphocytes from systemic lupus erythematosus patients with lymphopenia. *Lupus* **15**, 600-605 (2006).

115. M. Kawano, T. Seya, I. Koni, H. Mabuchi, Elevated serum levels of soluble membrane cofactor protein (cd46, mcp) in patients with systemic lupus erythematosus (sle). *Clin Exp Immunol* **116**, 542-546 (1999).

116. U. Ellinghaus, A. Cortini, C. L. Pinder, G. Le Friec, C. Kemper, T. J. Vyse, Dysregulated cd46 shedding interferes with th1-contraction in systemic lupus erythematosus. *Eur J Immunol* **47**, 1200-1210 (2017).

117. M. H. Tseng, S. H. Lin, C. Y. Wu, H. P. Chien, H. Y. Yang, Y. C. Chen, Y. C. Chou, J. L. Huang, Serum complement factor i is associated with disease activity of systemic lupus erythematosus. *Oncotarget* **9**, 8502-8511 (2018).

118. F. M. Wang, D. Song, Y. Pang, Y. Song, F. Yu, M. H. Zhao, The dysfunctions of complement factor h in lupus nephritis. *Lupus* **25**, 1328-1340 (2016).

119. J. Zhao, H. Wu, M. Khosravi, H. Cui, X. Qian, J. A. Kelly, K. M. Kaufman, C. D. Langefeld, A. H. Williams, M. E. Comeau, . . . B. P. Tsao, Association of genetic variants in complement factor h and factor h-related genes with systemic lupus erythematosus susceptibility. *PLoS Genet* **7**, e1002079 (2011).

120. L. Bao, M. Haas, R. J. Quigg, Complement factor h deficiency accelerates development of lupus nephritis. *J Am Soc Nephrol* **22**, 285-295 (2011).

121. K. Steinsson, K. Erlendsson, H. Valdimarsson, Successful plasma infusion treatment of a patient with c2 deficiency and systemic lupus erythematosus: Clinical experience over forty-five months. *Arthritis Rheum* **32**, 906-913 (1989).

122. R. F. Olsson, S. Hagelberg, B. Schiller, O. Ringden, L. Truedsson, A. Ahlin, Allogeneic hematopoietic stem cell transplantation in the treatment of human c1q deficiency: The karolinska experience. *Transplantation* **100**, 1356-1362 (2016).

123. M. C. Pickering, M. Ismajli, M. B. Condon, N. McKenna, A. E. Hall, L. Lightstone, H. Terence Cook, T. D. Cairns, Eculizumab as rescue therapy in severe resistant lupus nephritis. *Rheumatology (Oxford, England)* **54**, 2286-2288 (2015).

124. R. Coppo, L. Peruzzi, A. Amore, S. Martino, L. Vergano, I. Lastauka, A. Schieppati, M. Noris, P. A. Tovo, G. Remuzzi, Dramatic effects of eculizumab in a child with diffuse proliferative lupus nephritis resistant to conventional therapy. *Pediatric nephrology (Berlin, Germany)* **30**, 167-172 (2015).

125. D. Wang, J. Li, Y. Zhang, M. Zhang, J. Chen, X. Li, X. Hu, S. Jiang, S. Shi, L. Sun, Umbilical cord mesenchymal stem cell transplantation in active and refractory systemic lupus erythematosus: A multicenter clinical study. *Arthritis Res Ther* **16**, R79 (2014).

126. H. Ma, C. Liu, B. Shi, Z. Zhang, R. Feng, M. Guo, L. Lu, S. Shi, X. Gao, W. Chen, L. Sun, Mesenchymal stem cells control complement c5 activation by factor h in lupus nephritis. *EBioMedicine* **32**, 21-30 (2018).

127. J. C. Jennette, P. H. Nachman, Anca glomerulonephritis and vasculitis. *Clin J Am Soc Nephrol* **12**, 1680-1691 (2017).

128. E. C. Hagen, M. R. Daha, J. Hermans, K. Andrassy, E. Csernok, G. Gaskin, P. Lesavre, J. Ludemann, N. Rasmussen, R. A. Sinico, A. Wiik, F. J. van der Woude, Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. Ec/bcr project for anca assay standardization. *Kidney Int* **53**, 743-753 (1998).

129. H. Xiao, P. Heeringa, P. Hu, Z. Liu, M. Zhao, Y. Aratani, N. Maeda, R. J. Falk, J. C. Jennette, Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* **110**, 955-963 (2002).

130. R. J. Falk, R. S. Terrell, L. A. Charles, J. C. Jennette, Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci U S A* **87**, 4115-4119 (1990).

131. P. Brogan, D. Eleftheriou, Vasculitis update: Pathogenesis and biomarkers. *Pediatr Nephrol* **33**, 187-198 (2018).

132. R. Syed, A. Rehman, G. Valecha, S. El-Sayegh, Pauci-immune crescentic glomerulonephritis: An anca-associated vasculitis. *Biomed Res Int* **2015**, 402826 (2015).

133. H. Xiao, A. Schreiber, P. Heeringa, R. J. Falk, J. C. Jennette, Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol* **170**, 52-64 (2007).

134. A. Schreiber, H. Xiao, J. C. Jennette, W. Schneider, F. C. Luft, R. Kettritz, C5a receptor mediates neutrophil activation and anca-induced glomerulonephritis. *J Am Soc Nephrol* **20**, 289-298 (2009).

135. M. A. C. Wester Trejo, L. A. Trouw, I. M. Bajema, The role of complement in antineutrophil cytoplasmic antibody-associated vasculitis. *Curr Opin Rheumatol* **31**, 3-8 (2019).

136. D. Miao, D. Y. Li, M. Chen, M. H. Zhao, Platelets are activated in anca-associated vasculitis via thrombin-pars pathway and can activate the alternative complement pathway. *Arthritis Res Ther* **19**, 252 (2017).

137. S. F. Chen, F. M. Wang, Z. Y. Li, F. Yu, M. H. Zhao, M. Chen, Plasma complement factor h is associated with disease activity of patients with anca-associated vasculitis. *Arthritis Res Ther* **17**, 129 (2015).

138. S. F. Chen, F. M. Wang, Z. Y. Li, F. Yu, M. Chen, M. H. Zhao, The functional activities of complement factor h are impaired in patients with anca-positive vasculitis. *Clin Immunol* **175**, 41-50 (2017).

139. S. F. Chen, F. M. Wang, Z. Y. Li, F. Yu, M. Chen, M. H. Zhao, Myeloperoxidase influences the complement regulatory activity of complement factor h. *Rheumatology (Oxford)* **57**, 2213-2224 (2018).

140. S. F. Chen, F. M. Wang, Z. Y. Li, F. Yu, M. Chen, M. H. Zhao, Complement factor h inhibits anti-neutrophil cytoplasmic autoantibody-induced neutrophil activation by interacting with neutrophils. *Front Immunol* **9**, 559 (2018).

141. D. Huugen, A. van Esch, H. Xiao, C. J. Peutz-Kootstra, W. A. Buurman, J. W. Tervaert, J. C. Jennette, P. Heeringa, Inhibition of complement factor c5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int* **71**, 646-654 (2007).

142. H. Xiao, D. J. Dairaghi, J. P. Powers, L. S. Ertl, T. Baumgart, Y. Wang, L. C. Seitz, M. E. Penfold, L. Gan, P. Hu, B. Lu, N. P. Gerard, C. Gerard, T. J. Schall, J. C. Jaen, R. J. Falk, J. C. Jennette, C5a receptor (cd88) blockade protects against mpo-anca gn. *J Am Soc Nephrol* **25**, 225-231 (2014).

143. P. Bekker, D. Dairaghi, L. Seitz, M. Leleti, Y. Wang, L. Ertl, T. Baumgart, S. Shugarts, L. Lohr, T. Dang, S. Miao, Y. Zeng, P. Fan, P. Zhang, D. Johnson, J. Powers, J. Jaen, I. Charo, T. J. Schall, Characterization of pharmacologic and pharmacokinetic properties of ccx168, a potent and selective orally administered complement 5a receptor inhibitor, based on preclinical evaluation and randomized phase 1 clinical study. *PLoS One* **11**, e0164646 (2016).

144. D. R. W. Jayne, A. N. Bruchfeld, L. Harper, M. Schaier, M. C. Venning, P. Hamilton, V. Burst, F. Grundmann, M. Jadoul, I. Szombati, V. Tesar, M. Segelmark, A. Potarca, T. J. Schall, P. Bekker, Randomized trial of c5a receptor inhibitor avacopan in anca-associated vasculitis. *Journal of the American Society of Nephrology : JASN* **28**, 2756-2767 (2017).

CHAPTER 3

Inverse correlation between serum complement component C1q levels and whole blood type-1 interferon signature in active tuberculosis and QuantiFERON-positive uveitis: implications for diagnosis

Benjamin Schrijver*[1], Douwe J. Dijkstra*[2], Nicole V. Borggreven[2], Rina La Distia Nora[3], Erika Huijser[4], Marjan A. Versnel[4], P. Martin van Hagen[1,5], Simone A. Joosten[6], Leendert A. Trouw[2], Willem A. Dik[1,5]

1. Department of Immunology, Laboratory Medical Immunology, Erasmus MC University Medical Center Rotterdam, Rotterdam, the Netherlands
2. Department of Immunology, Leiden University Medical Center, Leiden, the Netherlands
3. Department of Ophthalmology, Faculty of Medicine, University of Indonesia and Cipto Mangunkusumo Hospital, Jakarta, Indonesia
4. Department of Immunology, Erasmus MC University Medical Center Rotterdam, Rotterdam, the Netherlands
5. Department of Internal Medicine, Division Clinical Immunology, Erasmus MC University Medical Center Rotterdam, Rotterdam, the Netherlands
6. Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands

* Shared first author

Clinical & Translational Immunology (2020), doi: *10.1002/cti2.1196*

Abstract

To examine the relation between serum C1q levels and blood type-1 interferon signature (type-1 IFN signature) in active pulmonary tuberculosis (APTB) and to determine whether combined measurement of serum C1q and type-1 IFN signature may add to the diagnosis of QuantiFERON (QFT)-positive patients with uveitis of unknown cause. C1q was determined (ELISA) in serum from two distinct Indonesian cohorts, and in total, APTB (n = 72), QFT-positive uveitis of unknown etiology (n = 58), QFT-negative uveitis (n = 51) patients and healthy controls (HC; n = 73) were included. The type-1 IFN signature scores were previously determined. Serum C1q was higher in APTB than HC ($p < 0.001$). APTB patients with uveitis had higher serum C1q than APTB patients without uveitis ($p = 0.0207$). Serum C1q correlated inversely with type-1 IFN signature scores in APTB ($p = 0.0036$, $r^2 = 0.3526$), revealing that these biomarkers for active TB disease can be mutually exclusive. Stratification of QFT-positive patients with uveitis of unknown cause, by serum C1q and type-1 IFN signature, yielded four groups with different likelihood of suffering from active TB uveitis. Serum C1q is elevated in APTB, especially in those cases with uveitis. We propose that combined measurement of blood type-1 IFN signature and serum C1q may provide added value in the diagnosis of active TB disease. Combined measurement of type-1 IFN signature and serum C1q in QFT-positive patients without signs of active TB disease, but suffering from uveitis of unknown cause, may be of help to identify cases with low or high likelihood of having active TB uveitis, which may facilitate clinical management decisions.

Introduction

Tuberculosis (TB) is an airborne infectious disease caused by Mycobacterium tuberculosis (*Mtb*) bacilli [1]. To date, TB remains a major global health threat and leading cause of death from a single infectious agent. The World Health Organization (WHO) estimates that a quarter of the world's population is infected with *Mtb*. Most of these individuals remain asymptomatically latently infected (LTBI), while ~ 5–10% develop clinically active TB disease during their lifetime [2]. The WHO estimated that ~ 10 million new TB cases emerged in 2018, with increasing TB notifications especially in India and Indonesia [2].

Although primarily a pulmonary pathogen, *Mtb* can disseminate from the primary focus of infection throughout the body via blood and lymphatic system, infecting other organs, including the eyes [3]. Ocular involvement has a prevalence between ~1.5% and 18% in pulmonary TB patients, but may also occur without pulmonary involvement and/or other signs of TB [4-9]. Timely diagnosis of active TB infection is crucial as it contributes to swift treatment initiation and thereby pathogen eradication, minimizing disease burden and curtailing *Mtb* transmission [2].

Sputum smear microscopy and culture-based methods are the standard diagnostic laboratory tests for active TB, but PCR-based techniques are also used to directly identify the pathogen [10-12]. All these tests are hampered by sensitivity and/or specificity issues and are often time consuming. Initiation of an adaptive immune response after *Mtb* exposure usually results in memory T-helper (Th)1-cell formation. These Th1 cells become activated upon re-exposure to *Mtb* antigens, which is typically exploited by in vitro interferon gamma (IFN-γ) release assays (e.g. QuantiFERON-TB Gold test; QFT) or tuberculin skin test (TST). Although these tests provide evidence of existing immunological memory towards *Mtb*, by no means do they discriminate between active TB disease and LTBI. The diagnosis of ocular TB is especially difficult to achieve because access to intraocular tissues is limited. Moreover, only a few *Mtb* bacilli may invade the intraocular environment, where they especially infect the retinal pigment epithelium (RPE) [13-15]. Yet, rapid initiation of anti-tuberculosis treatment (ATT) in TB-associated uveitis is important as substantial vision loss, or even blindness, can occur if left untreated [16, 17]. Especially in high TB-endemic areas, the association between QFT positivity and uveitis might be coincidental. Therefore, it is important to identify those QFT-positive uveitis patients suffering from active TB disease, especially when there are no other signs of active TB infection. Consequently, QFT-positive uveitis patients with LTBI could be prevented from undergoing overzealous ATT treatment. Altogether, this indicates the urgent need for biomarkers that discriminate active TB from LTBI, especially in those areas

where TB is highly endemic.

Several reports have indicated that patients suffering from active TB disease display a peripheral blood transcriptome that is highly enriched for type-1 IFN-stimulated genes (ISGs) [18-21]. In line with this, we recently reported a whole blood transcriptional signature of 10 ISGs (type-1 IFN signature) that was associated with active pulmonary TB (APTB) in Indonesian patients. In addition, we demonstrated that this type-1 IFN signature stratified QFT-positive patients with uveitis of undetermined cause into distinct groups. We proposed that type-1 IFN signature-positive patients were more likely to suffer from active TB-associated uveitis [22]. Furthermore, increased expression of mRNA for complement component C1q has been described in peripheral blood cells from active TB patients [23-25]. More recently, Lubbers *et al.* reported elevated serum C1q levels in APTB as compared to LTBI, healthy controls, and disease controls [23]. Consequently, serum C1q was proposed as biomarker to detect active TB disease. However, Lubbers *et al.* did not explore serum C1q levels in relation to TB-associated uveitis. Also, no correlations with peripheral blood type-1 IFN signatures were determined. However, the latter is of special interest as C1q has been shown to attenuate IFN-α production by peripheral blood mononuclear cells [26, 27].

Therefore, in our current study, we examined the following: (1) serum C1q levels in two independently collected cohorts of Indonesian patients with APTB and healthy controls, (2) the correlation between serum C1q levels and the type-1 IFN signature score in active TB and systemic autoimmune diseases [primary Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc)], and (3) whether the combination of serum C1q level and peripheral blood type-1 IFN signature may improve stratification of QFT-positive patients with uveitis of unknown cause, into groups highly likely, or less likely, to suffer from active TB-associated uveitis.

Results

Increased serum C1q levels in active pulmonary TB patients

In both independently collected Indonesian cohorts, serum C1q levels were significantly increased in APTB patients ($p < 0.001$) as compared to healthy controls (Figure 1). Furthermore, serum C1q levels were significantly increased in APTB patients from cohort 1 ($p = 0.0305$) as compared to cohort 2, while no statistically significant difference in serum C1q level was observed between the healthy control groups (Figure 1). The capacity of serum C1q to discriminate APTB from healthy controls was analyzed using receiver operating characteristic (ROC) curve analysis on all APTB cases (n = 72) versus

all healthy controls (n = 73) and revealed an area under the curve of 0.786 ($p < 0.001$; 95% CI 0.712–0.859; Figure 2). Diagnostic performance, as calculated by maximum Youden's index, revealed a sensitivity of 65.3% and specificity of 79.5%, when a cut-off value for serum complement C1q of ≥ 271.1 µg/ml was used (Supplementary table 2).

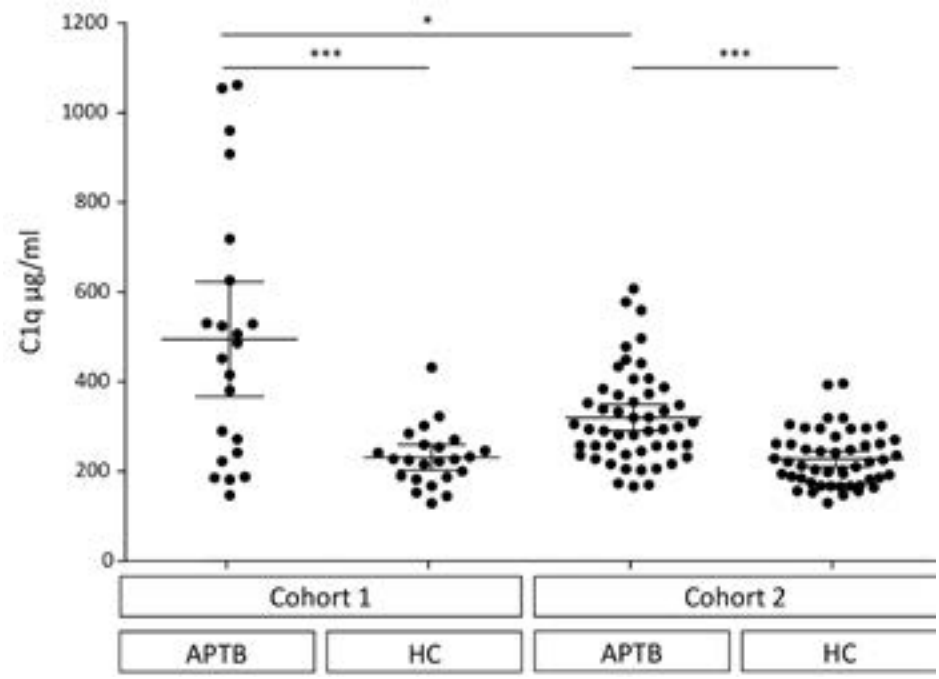
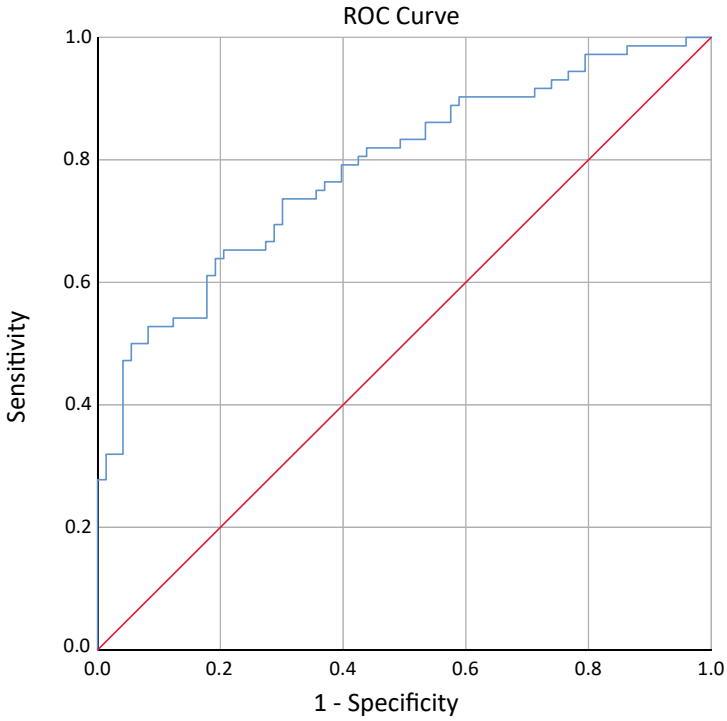


Figure 1. Increased serum C1q levels in active pulmonary tuberculosis (APTB). Comparison of serum C1q levels in APTB patients and healthy controls from Indonesian cohort 1 (left, APTB n = 22, healthy controls n = 23) and Indonesian cohort 2 (right, APTB n = 50, healthy controls n = 50). APTB, active pulmonary tuberculosis; HC, healthy control. Horizontal lines represent the group mean, and error bars indicate the 95% confidence interval (CI). Statistical analysis was performed on the transformed data set in GraphPad Prism 5.0, and Welch's t-test was used to compare groups. *p < 0.05, ***p < 0.001.



Area Under the Curve				
Area	Std. Error	Asymptotic Sig	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.786	0.038	0.000	0.712	0.859

Figure 2. Receiver operating characteristics curve analysis of the ability of serum C1q to distinguish APTB patients (cohort 1 + 2; n = 72) from healthy controls (cohort 1 + 2; n = 73). Statistical analysis was performed on the transformed data set in SPSS 25.

Inverse correlation between serum C1q levels and peripheral blood type-1 IFN signature scores in active pulmonary TB patients

In APTB patients (cohort 1, n = 22), serum C1q levels displayed a significant inverse correlation with the type-1 IFN signature scores (r^2 = 0.3526; p = 0.0036, Figure 3A). Substantial subsets of patients with pSS, SLE and SSc also display a positive type-1 IFN signature [28-31]. Therefore, to determine whether the inverse correlation between serum C1q and peripheral blood type-1 IFN gene signature might be TB specific or represents a more general phenomenon, we also determined the relation between serum C1q levels and peripheral blood type-1 IFN signature scores in pSS, SLE and SSc patients. Serum C1q did not show any correlation with the peripheral blood type-1 IFN signature scores in any of these systemic autoimmune diseases (Supplementary Figure 1).

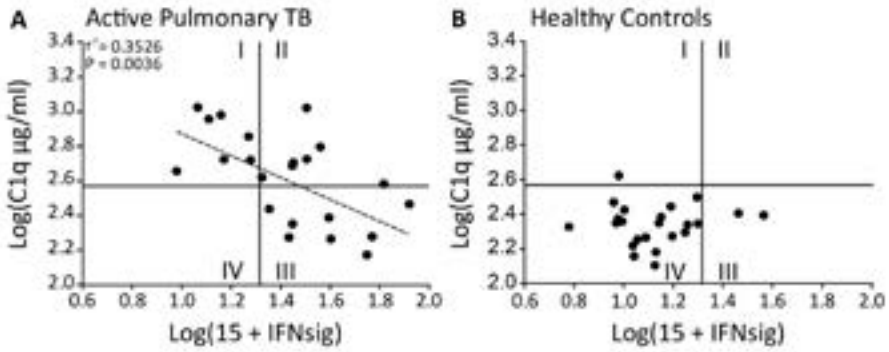


Figure 3. Correlation between serum C1q levels and whole blood transcriptional signature score, consisting of 10 type-1 interferon-stimulated genes (type-1 IFN signature). The vertical line represents the previously reported22 type-1 IFN signature score cut-off value for active TB. The horizontal line represents the serum C1q cut-off value based on the 95% reference interval for healthy controls (cohort 1 + 2; n = 73). (A) Inverse correlation between serum C1q level and type-1 IFN signature score in APTB patients (cohort 1; n = 22). None of the patients located to the left lower quadrant IV (normal serum C1q and a negative type-1 IFN signature). (B) No correlation between serum C1q levels and type-1 IFN signature score in healthy controls (cohort 1, n = 23). Twenty of the 23 healthy controls located to the lower left quadrant IV (normal serum C1q and a negative type-1 IFN signature). Statistical analysis was performed in GraphPad Prism 5.0, and correlation coefficient was determined with Pearson's r test.

On the basis of all Indonesian healthy controls (n = 73, cohorts 1 and 2), the upper limit of the 95% reference range was used as a cut-off value for serum C1q levels. This yielded a cut-off value of 2.57 [log (C1q µg/ml)], indicated by the horizontal line in Figure 3. We also applied a log transformation on the previously reported type-1 IFN signature score cut-off value for positivity, yielding a value of 1.31, as indicated by the vertical line in Figure 3 [22]. This generated four quadrants based on serum C1q and type-1 IFN signature score. Most importantly, application of these quadrants clearly demonstrated that although both increased serum C1q level and a positive blood type-1 IFN signature have been suggested as potential biomarker for active TB, they can be mutually exclusive [Figure 3A: quadrants I (elevated serum C1q and negative type-1 IFN signature score) and III (normal serum C1q and positive type-1 IFN signature score)]. Twenty out of 23 (87%) healthy controls located to quadrant IV (normal serum C1q and negative type-1 IFN gene signature; Figure 3B), while none of the APTB cases located to this quadrant (Figure 3A).

Increased serum C1q levels in QFT-positive patients with uveitis of unknown cause

C1q levels were significantly elevated in serum from QFT-positive patients with uveitis of unknown etiology (n = 58) as compared to QFT-negative uveitis patients (p <

0.001) and healthy controls ($p < 0.001$). Serum C1q levels were comparable between QFT-negative uveitis patients and healthy controls (Figure 4). Comparison between infectious and non-infectious uveitis within the QFT-negative uveitis group revealed no difference in serum C1q levels (Figure 5A). Interestingly, APTB patients with uveitis displayed significantly higher serum C1q levels than APTB patients without uveitis ($p = 0.0207$; Figure 5B). Furthermore, APTB patients with uveitis had significantly ($p < 0.001$) elevated serum C1q levels as compared to QFT-negative patients suffering from uveitis because of infection with the obligate intracellular parasite Toxoplasma gondii. Serum C1q levels in Toxoplasmosis-associated uveitis patients were comparable to levels in healthy controls (Figure 5C).

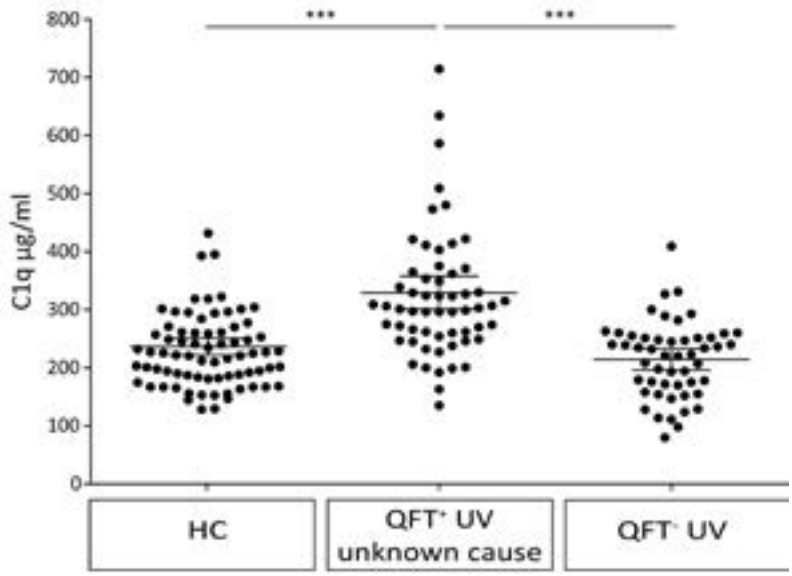


Figure 4. Increased serum C1q levels are associated with QuantiFERON-Tb Gold (QFT)-positive uveitis of unknown cause. Comparison of serum C1q levels in QFT-positive patients with uveitis of unknown cause (cohort 1; n = 58), QFT-negative patients with uveitis (cohort 1; n = 51) and healthy controls (cohort 1 + 2; n = 73). UV, uveitis. Horizontal lines represent the group mean, and error bars indicate the 95% CI. Statistical analysis was performed on the transformed data set in SPSS 25, and multiple comparison was performed by analysis of variance (ANOVA) followed by Dunnett's T3 post hoc testing. ***$p < 0.001$.

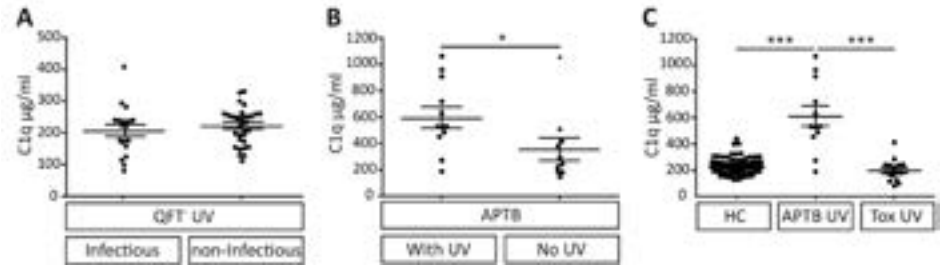


Figure 5. Increased serum C1q levels in APTB patients with uveitis. (A) Comparison of serum C1q levels between infectious and non-infectious uveitis amongst QFT-negative patients (cohort 1; n = 51). (B) Comparison of serum C1q levels in APTB patients with uveitis (cohort 1; n = 12) and APTB patients without uveitis (cohort 1; n = 10). (C) Comparison between serum C1q levels in APTB patients with uveitis (cohort 1; n = 12), QFT-negative patients with Toxoplasmosis gondii-associated uveitis (cohort 1; n = 16) and healthy controls (cohort 1 + 2; n = 73). Tox, Toxoplasmosis gondii. Horizontal lines represent the group mean, and error bars indicate the 95% CI. Statistical analysis was performed on the transformed data set in SPSS 25, and multiple comparison was performed by ANOVA followed by Dunnett's T3 post hoc testing. *$p < 0.05$, ***$p < 0.001$.

The combination of serum C1q and peripheral blood type-1 IFN signature distinguishes four distinct groups within QFT-positive uveitis patients

Applying the combination of serum C1q and the type-1 IFN signature score cut-off values to the QFT-positive patients with uveitis of unknown cause (n = 58) differentiated these patients into four distinct groups (Figure 6). The majority of patients (n = 30; 52%) located to quadrant IV (normal serum C1q and a negative type-1 IFN signature), 17 patients (29%) located to quadrant III (normal serum C1q and a positive type-1 IFN signature), six patients (10%) located to quadrant II (elevated serum C1q and a positive type-1 IFN signature), and five patients (9%) located to quadrant I (elevated serum C1q and a negative type-1 IFN signature).

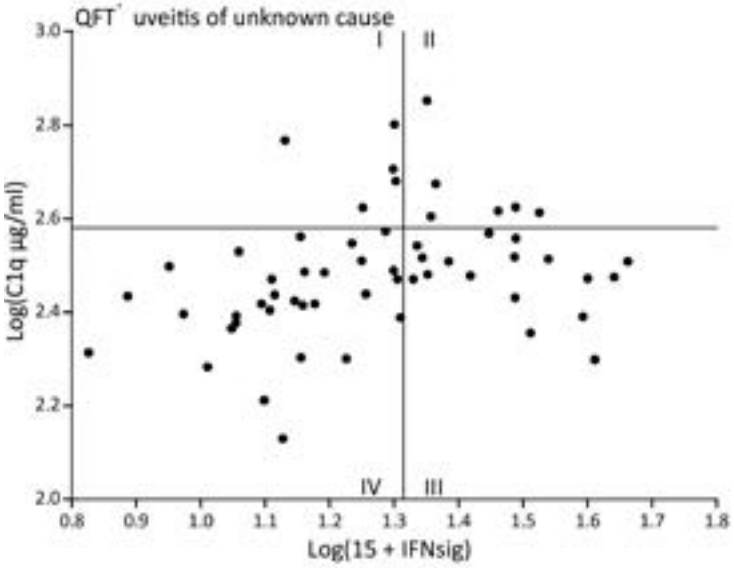


Figure 6. Application of serum C1q and type-1 IFN signature score to QFT-positive patients with uveitis of unknown cause. The type-1 IFN signature score cut-off value is indicated by the vertical line, and the serum C1q level cut-off value is indicated by the horizontal line. From the QFT-positive patients with uveitis of unknown cause (cohort 1; n = 58), 30 patients located to quadrant IV (normal serum C1q and a negative type-1 IFN signature) and might be considered as unlikely to suffer from uveitis because of active TB. Five patients located to quadrant I, six patients located to quadrant II, and 17 patients located to quadrant III. The patients within quadrants I, II and III are more likely to suffer from uveitis because of active TB.

Discussion

In our current study, we established an association between APTB (with or without uveitis) and elevated serum C1q levels in two, independently collected, Indonesian cohorts. ROC analysis displayed the diagnostic value of serum C1q. Serum C1q diagnostic performance, as calculated by maximum Youden's index (0.4473), revealed a sensitivity of 65.3% and specificity of 79.5% to discriminate APTB patients from geographically matched healthy controls. These findings confirm the previous study by Lubbers *et al.* that reported increased serum C1q levels in APTB patients from distinct geographical locations (Italy, Gambia, Korea and South Africa) as compared to geographically matched healthy controls, LTBI and clinically relevant diseases (e.g. sarcoidosis, leprosy and pneumonia) [23]. Our study is limited in the sense that not all included healthy controls were examined for the presence of LTBI, which could have served as a separate control group. However, equal serum C1q levels in LTBI and healthy controls have previously been observed [23]. Therefore, our data support the notion that elevated serum C1q measurement may provide added value in the diagnosis of active TB infections.

We, and others, previously demonstrated that APTB is associated with overexpression of ISGs in peripheral blood cells and that this may represent a biomarker for active TB, progression from LTBI to active TB, or to monitor treatment efficacy [18-20, 22]. Interestingly, associations between C1q deficiency and exacerbated IFN-α responses have been reported [27]. Moreover, C1q was noted to inhibit immune complex-induced IFN-α production by plasmacytoid dendritic cells, while hepatitis C virus core protein was found to downregulate inflammatory cytokine production via C1q receptor (gC1qR) ligation [26, 27, 32]. These findings indicate that C1q-gC1qR interactions may regulate cytokine responses, including IFN-α. Therefore, we determined the relation between serum C1q levels and type-1 IFN signature scores in active TB disease, as both have been proposed as biomarker to identify active TB. We observed a clear inverse correlation between serum C1q levels and type-1 IFN signature scores in APTB patients, indicating that both these proposed biomarkers for active TB disease can be mutually exclusive. We propose that the combined measurement of type-1 IFN signature and serum C1q may provide added value in the diagnosis of active TB disease.

Positive blood type-1 IFN gene signatures also occur in substantial numbers of patients with systemic autoimmune disease, including pSS, SLE or SSc [28-31]. We did not observe a correlation between the disease-specific type-1 IFN gene signature score and serum C1q level in systemic autoimmune disease (pSS, SLE or SLE). Several studies did identify transcriptional differences, as well as similarities, in type-1 IFN-inducible gene signatures between various autoimmune diseases, TB, and normal immune responses, supporting the use of disease-specific IFN gene signatures [33, 34]. Thus, although the genes underlying the type-1 IFN gene signature differed between our TB cohort and the systemic autoimmune disease cohort, our data support a role for C1q in regulating type-1 IFN signaling during active TB disease. How this relates to disease pathology, duration, activity or progression is still unclear and should be investigated in future studies.

Interestingly, APTB patients with uveitis displayed higher serum C1q levels than APTB patients without uveitis. The exact reason for this is currently unknown. However, C1q can modulate T-cell responses by attenuating the activity of $CD8^+$ T cells, Th1 and Th17 cells and enhancing regulatory T-cell activity [35, 36]. Furthermore, serum analysis of rhesus macaques with pulmonary TB revealed a further increase in serum C1q level in those macaques that were unable to control disease, while levels remained stable at pre-infection levels in macaques that did control the infection [23, 37]. This further supports a potential immune dampening effect of C1q that hampers *Mtb* control (within the lungs), thereby leading to pulmonary disease progression and potential systemic *Mtb* spread. Moreover, increased serum C1q levels, during TB infection, are

accompanied by an increase in the expression of SERPING1, the main inhibitor of the C1-complex [23, 38]. This may suggest the presence of a TB-specific immune-escape mechanism in which the immune-suppressive effect of C1q on T-cell biology is achieved without increased classical complement pathway activation. At the same time, immune dampening by C1q might limit immunopathology, which might be beneficial at vital and vulnerable anatomical locations such as the eyes.

Previously, we did not observe increased expression of C1QA, C1QB or C1QC mRNA transcripts in *Mtb*-infected RPE cells [39]. This suggests that *Mtb*-infected RPE cells most likely do not directly contribute to the high serum C1q levels we observed in TB-associated uveitis. Potentially, RPE-derived molecules stimulate other cell types (e.g. monocytes, macrophages and/or dendritic cells) within and/or outside the eye to produce C1q. Transcriptional and immuno-histochemical analysis on enucleated eye structures of patients suffering from TB-associated uveitis would be of great interest to identify any C1q-producing cells in the eye.

Swift diagnosis of TB-associated uveitis is crucial to minimize disease burden and optimize treatment outcome [10]. However, QFT and TST do not discriminate between a current active *Mtb* infection and past exposure. Moreover, especially in TB-endemic countries, the association between a positive QFT and/or TST test with uveitis might be coincidental. In such areas, over-diagnosis of active TB-associated uveitis is a recognized problem that may result in overzealous treatment of QFT-positive uveitis patients with highly toxic anti-TB drugs [40]. This underlines the need for new biomarkers, or combinations thereof, to stratify especially QFT-positive patients with uveitis of unknown cause, into those that are less likely, or highly likely, to suffer from uveitis because of an active *Mtb* infection [41-44]. Previously, we proposed that QFT-positive uveitis patients with a negative type-1 IFN signature had a low risk of suffering from uveitis because of an active TB infection [22]. However, our current data indicate that active TB can exist in the context of a negative type-1 IFN signature, in the presence of elevated serum C1q levels. Therefore, based on our current finding, we propose that QFT-positive patients with uveitis of unknown cause, that have a negative type-1 IFN signature and normal serum C1q levels (quadrant IV, Figure 6), are least likely to suffer from uveitis because of an active TB infection. These patients might, for instance, suffer from autoimmune uveitis, either related or unrelated to past *Mtb* infection, or from uveitis of another (unidentified) cause [45, 46]. In contrast, TB-associated uveitis is highly likely in patients falling into one of the other quadrants (quadrants I, II and III; Figure 6). Combined type-1 IFN signature and serum C1q measurement could therefore aid in the diagnostic workup of QFT-positive uveitis patients to optimize treatment choice and potentially prevent unnecessary treatment with highly

toxic anti-TB drugs in a substantial number of patients (quadrant IV; Figure 6).

A limitation of our study is that we had no data on outcome/treatment response available. Such data would have been of additional value to further support our hypothesis that QFT-positive uveitis patients that fall into quadrants I, II or III are likely to display a beneficial response to ATT, while patients falling into quadrant IV most likely do not. Therefore, studies that explore the relation between peripheral blood type-1 IFN signature and serum C1q level in relation to the efficacy of ATT in QFT-positive uveitis patients are warranted.

In conclusion, in this study we confirm the recently published association between active TB disease and elevated serum C1q levels. Our study is the first to reveal an inverse correlation between serum C1q level and type-1 IFN signature score in active TB disease. Importantly, our data clearly demonstrate that increased serum C1q levels or a positive type-1 IFN signature are individually not sufficient to detect active TB disease. We propose that the combined measurement of peripheral blood type-1 IFN signature and serum C1q level is of added value in the diagnosis of active TB disease. This is further supported by ROC analysis on cohort 1, where predicted probabilities calculated by binary logistic regression on the combined measurement of serum C1q and type-1 IFN signature score yielded a sensitivity of 100% and specificity of 87%, thus outperforming the diagnostic accuracy of serum C1q or type-1 IFN signature score alone (Supplementary Figure 2). However, additional studies using higher numbers of patients are required to confirm this observation. Moreover, we expect that combined measurement of both these biomarkers will improve the stratification of patients that are suspected of active TB-associated uveitis into groups with either a low or high risk of having uveitis because of an active TB infection. Such a stratification could form the basis for future diagnostic and treatment studies in QFT-positive uveitis patients.

Materials and methods

Patients and controls

Sera from three previously collected cohorts, two TB cohorts from Indonesia (herein designated as cohort 1 [47] and 2 [48]) and one cohort consisting of systemic autoimmune patients from mainly Caucasian origin (herein designated as cohort 3 [28-30]), were included in this study. All individuals from cohorts 1 and 3 and all TB patients from cohort 2 were HIV negative, and healthy controls from cohort 2 were largely untested. A detailed description of the patient cohorts is given in Table 1. All included individuals provided their written informed consent. The study was

approved by the local medical ethics committees of the Faculty of Medicine University of Indonesia (cohort 1, FMUI: 268/H2.F1/ETIK/2014), The Eijkman Institute research ethics committee, Jakarta (cohort 2), and Erasmus MC, University Medical Center, Rotterdam, the Netherlands (cohort 3, MEC-2011-116). This study was conducted in coherence with the tenets of the Declaration of Helsinki.

Table 1. Patient characteristics. APTB, active pulmonary tuberculosis; IFN, interferon; QFT, QuantiFERON-TB Gold test; SD, standard deviation.

(Sub)Group	Mean Age (SD)	Male-to-Female ratio	Sputum positive	QFT positive (> 0.35 IU/ml)	Type I IFN signature score positive (> 5.6)
Cohort 1 (n = 154)					
Healthy Control (n = 23)	31.0 (9.4)	0.4	n/a	0/23	2/23
APTB w/o uveitis (n = 10)	41.3 (15.7)	2.3	10/10	7/10	10/10
APTB-assoc. uveitis (n = 12)	42.0 (17.2)	2.0	2/12	10/12	5/12
QFT-positive Uveitis of unknown etiology (n = 58)	46.3 (13.2)	0.4	n/a	58/58	23/58
QFT-negative Uveitis (n = 51)	39.9 (16.5)	1.6	n/a	0/51	21/58
Cohort 2 (n = 100)					
Healthy Control (n = 50)	34.6 (12.6)	0.9	0/50	n/a	n/a
APTB uveitis unknown (n = 50)	31.4 (9.5)	2.1	50/50	n/a	n/a
Cohort 3 (n = 139)					
Primary Sjögren's syndrome (n = 86)	60.2 (13.2)	0.1	n/a	n/a	n/a
Systemic lupus erythematosus (n = 30)	46.8 (13.6)	0.1	n/a	n/a	n/a
Systemic sclerosis (n = 23)	59.5 (10.9)	0.2	n/a	n/a	n/a

Tuberculosis, QFT-positive uveitis and QFT-negative uveitis

Cohort 1 was previously described and originates from Jakarta (n = 131 patients and n = 23 healthy controls) [47]. Cohort 1 contained 10 sputum smear-positive APTB patients without uveitis, 12 clinically diagnosed APTB patients with uveitis of whom two were sputum smear positive, 58 QuantiFERON-Tb Gold test (QFT)-positive (> 0.35 IU/ml) uveitis patients without any signs of active TB and no alternative determined uveitis cause, 51 QFT-negative uveitis patients [infectious origin (n = 19), related to systemic disease (n = 5), ocular syndromes (n = 12) or uveitis of unknown etiology (n = 15)], and 23 healthy Indonesian controls. Cohort 2 also originates from Jakarta and consisted of 50, sputum smear and culture positive, APTB patients and 50 healthy Indonesian controls. Diagnostic information concerning the presence of uveitis in these patients was not available [48].

Systemic autoimmune disease

Cohort 3 consists of sera from patients with pSS (n = 86), SLE (n = 30) and SSc (n = 23) that were included at the Erasmus MC, University Medical Center, Rotterdam, the Netherlands.

Serum C1q ELISA

Serum C1q levels were determined by ELISA, as described previously [23, 49]. In short, MaxiSorp (Nunc, Rochester, NY, USA) plates were coated overnight at 4°C with mouse anti-human C1q antibody (Department of Nephrology, Leiden University Medical Center, the Netherlands) in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6). The next day, plates were washed and blocked with PBS/1% BSA for 1 h at 37°C. After washing, the plates were incubated for 1 h at 37°C with serum samples serially diluted in PBS/1% BSA/0.05% Tween. Subsequently, the plates were washed and incubated (1 h, 37°C) with rabbit anti-human C1q antibody (DAKO, Jena, Germany). Hereafter, the plates were washed and incubated (1 h, 37°C) with horseradish peroxidase (HRP)-labelled goat-anti-rabbit antibody (DAKO). As substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Sigma, Saint Louis, MO, USA) was used to optically visualize HRP activity, which was quantified by measuring the optical density at 415 nm. Wash steps were performed with PBS/0.05% Tween. All serum sample measurements within the linear range of the reference curve were averaged, and reference curve dilutions were analyzed in duplicate.

Peripheral blood type-1 IFN gene signatures

From patients and healthy controls included in cohort 1, the TB-related peripheral blood type-1 IFN gene signature scores were previously determined in blood obtained at the same time as serum used for C1q analysis in our current study [22]. In addition, from cohort 3, the systemic autoimmune disease-related peripheral blood type-1 IFN signature scores were available and determined as previously described [28-30]. Genes comprising the disease-related type-1 IFN signatures, as well as the formula used to calculate the type-1 IFN signature score, are provided in Supplementary table 1.

Statistical analysis

Data distribution of all groups was determined with the D'Agostino–Pearson normality test. As most of the data were not normally distributed within the groups, a log transformation was applied on the complete data set. C1q values were transformed by log (C1q µg/ml), while the type-1 IFN signature scores were transformed by log (15 + type-1 IFN signature).

Comparison between two groups was performed with an unpaired Student's t-test. If group variances differed significantly (F-test), an unpaired Welch's t-test was used instead. Correlations were determined with Pearson's r test. Multiple comparison testing was performed by analysis of variance (ANOVA) followed by Dunnett's T3 post hoc testing. Statistical analysis was performed in GraphPad Prism 5.0 and SPSS 25. A p-value < 0.05 was considered significant.

Acknowledgements

We are grateful to all participants who donated blood for these studies. We acknowledge the efforts of Professor Drs R. Sitompul, M. Susiyanti, L. Edwar, S. Sjamsoe (Department of Internal Medicine, Respirology and Critical Illness Division, University of Indonesia & Cipto Mangunkusumo Hospital Kirana, Jakarta, Indonesia), Dr G. Singh (Department of Internal Medicine, Respirology and Critical Illness Division, University of Indonesia & Cipto Mangunkusumo Hospital Kirana, Jakarta, Indonesia) and Dr R.R. Diah Handayani (Department of Pulmonology, Persahabatan Hospital, Jakarta, Indonesia) for patient inclusion and sample collection of cohort 1. We also acknowledge Dr B. Alisjahbana (Department of Internal Medicine, Hasan Sadikin General Hospital, Bandung, Indonesia, & Infectious Disease Research Center, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia) and his team for patient inclusion and sample collection of cohort 2.

Conflict of Interest

The authors have no conflicts of interests to disclose.

References

1. K. Dheda, C. E. Barry, 3rd, G. Maartens, Tuberculosis. *Lancet* **387**, 1211-1226 (2016).
2. WHO, Global tuberculosis report 2019. *https://apps.who.int/iris/bitstream/handle/10665/329368/9789241565714-eng.pdf?ua=1*.
3. J. Barrios-Payan, M. Saqui-Salces, M. Jeyanathan, A. Alcantara-Vazquez, M. Castanon-Arreola, G. Rook, R. Hernandez-Pando, Extrapulmonary locations of mycobacterium tuberculosis DNA during latent infection. *J Infect Dis* **206**, 1194-1205 (2012).
4. H. C. Donahue, Ophthalmologic experience in a tuberculosis sanatorium. *Am J Ophthalmol* **64**, 742-748 (1967).
5. E. Bouza, P. Merino, P. Munoz, C. Sanchez-Carrillo, J. Yanez, C. Cortes, Ocular tuberculosis. A prospective study in a general hospital. *Medicine (Baltimore)* **76**, 53-61 (1997).
6. A. M. Mansour, R. Haymond, Choroidal tuberculomas without evidence of extraocular tuberculosis. *Graefes Arch Clin Exp Ophthalmol* **228**, 382-383 (1990).
7. S. Alvarez, W. R. McCabe, Extrapulmonary tuberculosis revisited: A review of experience at boston city and other hospitals. *Medicine (Baltimore)* **63**, 25-55 (1984).
8. L. Cimino, C. P. Herbort, R. Aldigeri, C. Salvarani, L. Boiardi, Tuberculous uveitis, a resurgent and underdiagnosed disease. *Int Ophthalmol* **29**, 67-74 (2009).
9. Y. Morimura, A. A. Okada, S. Kawahara, Y. Miyamoto, S. Kawai, A. Hirakata, T. Hida, Tuberculin skin testing in uveitis patients and treatment of presumed intraocular tuberculosis in japan. *Ophthalmology* **109**, 851-857 (2002).
10. M. Pai, M. A. Behr, D. Dowdy, K. Dheda, M. Divangahi, C. C. Boehme, A. Ginsberg, S. Swaminathan, M. Spigelman, H. Getahun, D. Menzies, M. Raviglione, Tuberculosis. *Nat Rev Dis Primers* **2**, 16076 (2016).
11. A. D. Harries, A. M. V. Kumar, Challenges and progress with diagnosing pulmonary tuberculosis in low- and middle-income countries. *Diagnostics (Basel)* **8** (2018).
12. A. N. Zeka, S. Tasbakan, C. Cavusoglu, Evaluation of the genexpert *Mtb*/rif assay for rapid diagnosis of tuberculosis and detection of rifampin resistance in pulmonary and extrapulmonary specimens. *J Clin Microbiol* **49**, 4138-4141 (2011).
13. N. A. Rao, S. Saraswathy, R. E. Smith, Tuberculous uveitis: Distribution of mycobacterium tuberculosis in the retinal pigment epithelium. *Arch Ophthalmol* **124**, 1777-1779 (2006).
14. O. Pandey, B. Paudyal, B. Basnyat, Gene-xpert: Diagnosis of pulmonary tuberculosis in a sputum smear negative patient. *J Nepal Health Res Counc* **17**, 125-127 (2019).
15. D. M. Albert, M. L. Raven, Ocular tuberculosis. *Microbiol Spectr* **4** (2016).
16. M. J. Thompson, D. M. Albert, Ocular tuberculosis. *Arch Ophthalmol* **123**, 844-849 (2005).
17. S. W. Dalvin LA, Intraocular manifestation of mycobacterium tuberculosis: A review of the literature. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases* **7**, 13-21 (2017).
18. M. P. Berry, C. M. Graham, F. W. McNab, Z. Xu, S. A. Bloch, T. Oni, K. A. Wilkinson, R. Banchereau, J. Skinner, R. J. Wilkinson, . . . A. O'Garra, An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* **466**, 973-977 (2010).
19. D. E. Zak, A. Penn-Nicholson, T. J. Scriba, E. Thompson, S. Suliman, L. M. Amon, H. Mahomed, M. Erasmus, W. Whatney, G. D. Hussey, . . . G. C. c. s. groups, A blood rna signature for tuberculosis disease risk: A prospective cohort study. *Lancet* **387**, 2312-2322 (2016).

20. T. H. Ottenhoff, R. H. Dass, N. Yang, M. M. Zhang, H. E. Wong, E. Sahiratmadja, C. C. Khor, B. Alisjahbana, R. van Crevel, S. Marzuki, M. Seielstad, E. van de Vosse, M. L. Hibberd, Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. *PLoS One* **7**, e45839 (2012).

21. L. Moreira-Teixeira, K. Mayer-Barber, A. Sher, A. O'Garra, Type i interferons in tuberculosis: Foe and occasionally friend. *J Exp Med* **215**, 1273-1285 (2018).

22. R. La Distia Nora, R. Sitompul, M. Bakker, M. A. Versnel, S. M. A. Swagemakers, P. J. van der Spek, M. Susiyanti, L. Edwar, S. Sjamsoe, G. Singh, R. D. Handayani, A. Rothova, P. M. van Hagen, W. A. Dik, Type 1 interferon-inducible gene expression in quantiferon gold tb-positive uveitis: A tool to stratify a high versus low risk of active tuberculosis? *PLoS One* **13**, e0206073 (2018).

23. R. Lubbers, J. S. Sutherland, D. Goletti, R. A. de Paus, C. H. M. van Moorsel, M. Veltkamp, S. M. T. Vestjens, W. J. W. Bos, L. Petrone, F. Del Nonno, . . . L. A. Trouw, Complement component c1q as serum biomarker to detect active tuberculosis. *Front Immunol* **9**, 2427 (2018).

24. Y. Cai, Q. Yang, Y. Tang, M. Zhang, H. Liu, G. Zhang, Q. Deng, J. Huang, Z. Gao, B. Zhou, C. G. Feng, X. Chen, Increased complement c1q level marks active disease in human tuberculosis. *PLoS One* **9**, e92340 (2014).

25. J. M. Cliff, J. S. Lee, N. Constantinou, J. E. Cho, T. G. Clark, K. Ronacher, E. C. King, P. T. Lukey, K. Duncan, P. D. Van Helden, G. Walzl, H. M. Dockrell, Distinct phases of blood gene expression pattern through tuberculosis treatment reflect modulation of the humoral immune response. *J Infect Dis* **207**, 18-29 (2013).

26. C. Lood, B. Gullstrand, L. Truedsson, A. I. Olin, G. V. Alm, L. Ronnblom, G. Sturfelt, M. L. Eloranta, A. A. Bengtsson, C1q inhibits immune complex-induced interferon-alpha production in plasmacytoid dendritic cells: A novel link between c1q deficiency and systemic lupus erythematosus pathogenesis. *Arthritis Rheum* **60**, 3081-3090 (2009).

27. D. M. Santer, B. E. Hall, T. C. George, S. Tangsombatvisit, C. L. Liu, P. D. Arkwright, K. B. Elkon, C1q deficiency leads to the defective suppression of ifn-alpha in response to nucleoprotein containing immune complexes. *J Immunol* **185**, 4738-4749 (2010).

28. E. Huijser, C. G. van Helden-Meeuwsen, N. Groot, I. L. A. Bodewes, M. J. Wahadat, M. W. J. Schreurs, P. L. A. van Daele, V. Dalm, J. A. M. van Laar, P. M. van Hagen, M. Waris, S. Kamphuis, M. A. Versnel, Mxa is a clinically applicable biomarker for type i interferon activation in systemic lupus erythematosus and systemic sclerosis. *Rheumatology (Oxford)* **58**, 1302-1303 (2019).

29. M. J. Wahadat, I. L. A. Bodewes, N. I. Maria, C. G. van Helden-Meeuwsen, A. van Dijk-Hummelman, E. C. Steenwijk, S. Kamphuis, M. A. Versnel, Type i ifn signature in childhood-onset systemic lupus erythematosus: A conspiracy of DNA- and rna-sensing receptors? *Arthritis Res Ther* **20**, 4 (2018).

30. Z. Brkic, M. A. Versnel, Type i ifn signature in primary sjogren's syndrome patients. *Expert Rev Clin Immunol* **10**, 457-467 (2014).

31. Z. Brkic, L. van Bon, M. Cossu, C. G. van Helden-Meeuwsen, M. C. Vonk, H. Knaapen, W. van den Berg, V. A. Dalm, P. L. Van Daele, A. Severino, N. I. Maria, S. Guillen, W. A. Dik, L. Beretta, M. A. Versnel, T. Radstake, The interferon type i signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high baff gene expression and high collagen synthesis. *Ann Rheum Dis* **75**, 1567-1573 (2016).

32. X. Song, Z. Yao, J. Yang, Z. Zhang, Y. Deng, M. Li, C. Ma, L. Yang, X. Gao, W. Li, J. Liu, L. Wei, Hcv core protein binds to gc1qr to induce a20 expression and inhibit cytokine production through mapks and nf-kappab signaling pathways. *Oncotarget* **7**, 33796-33808 (2016).

33. T. M. Aune, K. Maas, J. Parker, J. H. Moore, N. J. Olsen, Profiles of gene expression in human autoimmune disease. *Cell Biochem Biophys* **40**, 81-96 (2004).

34. K. Maas, S. Chan, J. Parker, A. Slater, J. Moore, N. Olsen, T. M. Aune, Cutting edge: Molecular portrait of human autoimmune disease. *J Immunol* **169**, 5-9 (2002).

35. G. S. Ling, G. Crawford, N. Buang, I. Bartok, K. Tian, N. M. Thielens, I. Bally, J. A. Harker, P. G. Ashton-Rickardt, S. Rutschmann, J. Strid, M. Botto, C1q restrains autoimmunity and viral infection by regulating cd8(+) t cell metabolism. *Science* **360**, 558-563 (2018).

36. E. V. Clarke, A. J. Tenner, Complement modulation of t cell immune responses during homeostasis and disease. *J Leukoc Biol* **96**, 745-756 (2014).

37. R. L. Karin Dijkman, Nicole V. Borggreven, Tom H.M. Ottenhoff, Simone A. Joosten, Leendert A. Trouw, Frank A.W. Verreck., Systemic and pulmonary c1q as biomarker of progressive disease in experimental non-human primate tuberculosis. *Scientific Reports* (2020 in press).

38. R. Lubbers, J. S. Sutherland, D. Goletti, R. A. de Paus, D. J. Dijkstra, C. H. M. van Moorsel, M. Veltkamp, S. M. T. Vestjens, W. J. W. Bos, L. Petrone, S. T. Malherbe, G. Walzl, K. A. Gelderman, G. H. Groeneveld, A. Geluk, T. H. M. Ottenhoff, S. A. Joosten, L. A. Trouw, Expression and production of the serping1-encoded endogenous complement regulator c1-inhibitor in multiple cohorts of tuberculosis patients. *Mol Immunol* **120**, 187-195 (2020).

39. R. La Distia Nora, K. V. Walburg, P. M. van Hagen, S. M. A. Swagemakers, P. J. van der Spek, E. Quinten, M. van Velthoven, T. H. M. Ottenhoff, W. A. Dik, M. C. Haks, Retinal pigment epithelial cells control early mycobacterium tuberculosis infection via interferon signaling. *Invest Ophthalmol Vis Sci* **59**, 1384-1395 (2018).

40. A. Gupta, A. Sharma, R. Bansal, K. Sharma, Classification of intraocular tuberculosis. *Ocul Immunol Inflamm* **23**, 7-13 (2015).

41. P. Elkington, M. Tebruegge, S. Mansour, Tuberculosis: An infection-initiated autoimmune disease? *Trends Immunol* **37**, 815-818 (2016).

42. A. Spratt, T. Key, A. J. Vivian, Chronic anterior uveitis following bacille calmette-guerin vaccination: Molecular mimicry in action? *J Pediatr Ophthalmol Strabismus* **45**, 252-253 (2008).

43. A. Garip, M. Diedrichs-Mohring, S. R. Thurau, C. A. Deeg, G. Wildner, Uveitis in a patient treated with bacille-calmette-guerin: Possible antigenic mimicry of mycobacterial and retinal antigens. *Ophthalmology* **116**, 2457-2462 e2451-2452 (2009).

44. L. L. Koth, O. D. Solberg, J. C. Peng, N. R. Bhakta, C. P. Nguyen, P. G. Woodruff, Sarcoidosis blood transcriptome reflects lung inflammation and overlaps with tuberculosis. *Am J Respir Crit Care Med* **184**, 1153-1163 (2011).

45. R. Tagirasa, S. Parmar, M. R. Barik, S. Devadas, S. Basu, Autoreactive t cells in immunopathogenesis of tb-associated uveitis. *Invest Ophthalmol Vis Sci* **58**, 5682-5691 (2017).

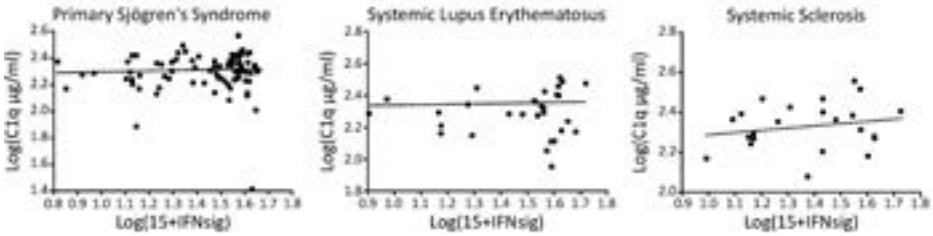
46. B. Schrijver, H. Hardjosantoso, J. Ten Berge, M. W. J. Schreurs, P. M. Van Hagen, R. A. Brooimans, A. Rothova, W. A. Dik, No evidence for circulating retina specific autoreactive t-cells in latent tuberculosis-associated uveitis and sarcoid uveitis. *Ocul Immunol Inflamm* 10.1080/09273948.2019.1698752, 1-7 (2020).

47. R. La Distia Nora, R. Sitompul, M. Bakker, M. Susiyanti, L. Edwar, S. Sjamsoe, G. Singh, M. P. van Hagen, A. Rothova, Tuberculosis and other causes of uveitis in indonesia. *Eye (Lond)* **32**, 546-554 (2018).

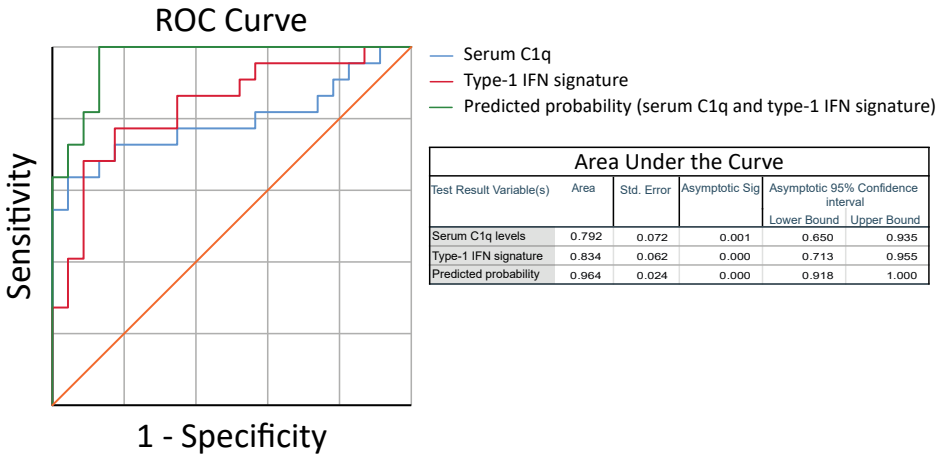
48. F. Vrieling, B. Alisjahbana, E. Sahiratmadja, R. van Crevel, A. C. Harms, T. Hankemeier, T. H. M. Ottenhoff, S. A. Joosten, Plasma metabolomics in tuberculosis patients with and without concurrent type 2 diabetes at diagnosis and during antibiotic treatment. *Sci Rep* **9**, 18669 (2019).

49. R. A. van Schaarenburg, C. Magro-Checa, J. A. Bakker, Y. K. Teng, I. M. Bajema, T. W. Huizinga, G. M. Steup-Beekman, L. A. Trouw, C1q deficiency and neuropsychiatric systemic lupus erythematosus. *Front Immunol* **7**, 647 (2016).

Supporting information



Supplementary Figure 1. Correlation between serum C1q levels and type-1 IFN signature in primary Sjögren's syndrome (n = 86), systemic lupus erythematosus (n = 30) and systemic sclerosis (n = 23) patients (cohort 3).



Area Under the Curve

Test Result Variable(s)	Area	Std. Error	Asymptotic Sig	Asymptotic 95% Confidence interval	
				Lower Bound	Upper Bound
Serum C1q levels	0.792	0.072	0.001	0.650	0.935
Type-1 IFN signature	0.834	0.062	0.000	0.713	0.955
Predicted probability	0.964	0.024	0.000	0.918	1.000

Coordinates of the Curve

Serum C1q levels					Type-1 IFN signature					Predicted probability				
Positive If Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J	Positive If Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J	Positive If Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J
1.1086	1.000	1.000	0.0000	0.0000	-0.2190	1.000	1.000	0.0000	0.0000	0.0000	1.000	1.000	0.0000	0.0000
2.1342	1.000	0.957	0.0435	0.0435	0.8719	1.000	0.957	0.0435	0.0435	0.0015	1.000	0.957	0.0435	0.0435
2.1627	1.000	0.913	0.0870	0.0870	0.9680	1.000	0.913	0.0870	0.0870	0.0024	1.000	0.913	0.0870	0.0870
2.1745	0.955	0.913	0.0870	0.0415	0.9763	1.000	0.870	0.1304	0.1304	0.0034	1.000	0.870	0.1304	0.1304
2.2034	0.955	0.870	0.1304	0.0850	0.9800	0.955	0.870	0.1304	0.0850	0.0054	1.000	0.826	0.1739	0.1739
2.2409	0.955	0.826	0.1739	0.1285	0.9820	0.955	0.826	0.1739	0.1285	0.0072	1.000	0.783	0.2174	0.2174
2.2594	0.909	0.826	0.1739	0.0830	0.9903	0.955	0.783	0.2174	0.1719	0.0087	1.000	0.739	0.2609	0.2609
2.2636	0.909	0.783	0.2174	0.1265	1.0015	0.955	0.739	0.2609	0.2154	0.0111	1.000	0.696	0.3043	0.3043
2.2696	0.864	0.783	0.2174	0.0810	1.0231	0.955	0.696	0.3043	0.2589	0.0131	1.000	0.652	0.3478	0.3478
2.2724	0.864	0.739	0.2609	0.1245	1.0430	0.955	0.652	0.3478	0.3024	0.0139	1.000	0.609	0.3913	0.3913
2.2758	0.818	0.739	0.2609	0.0791	1.0526	0.955	0.609	0.3913	0.3458	0.0234	1.000	0.565	0.4348	0.4348
2.2896	0.818	0.696	0.3043	0.1225	1.0624	0.955	0.565	0.4348	0.3893	0.0343	1.000	0.522	0.4783	0.4783
2.3170	0.818	0.652	0.3478	0.1660	1.0791	0.909	0.565	0.4348	0.3439	0.0377	1.000	0.478	0.5217	0.5217
2.3400	0.818	0.609	0.3913	0.2095	1.1015	0.909	0.522	0.4783	0.3874	0.0472	1.000	0.435	0.5652	0.5652
2.3465	0.818	0.565	0.4348	0.2530	1.1198	0.864	0.522	0.4783	0.3419	0.0694	1.000	0.391	0.6087	0.6087
2.3492	0.773	0.565	0.4348	0.2075	1.1318	0.864	0.478	0.5217	0.3854	0.0853	1.000	0.348	0.6522	0.6522
2.3536	0.773	0.522	0.4783	0.2510	1.1401	0.864	0.435	0.5652	0.4289	0.1172	1.000	0.304	0.6957	0.6957
2.3557	0.773	0.478	0.5217	0.2945	1.1504	0.864	0.391	0.6087	0.4723	0.1839	1.000	0.261	0.7391	0.7391
2.3606	0.773	0.435	0.5652	0.3379	1.1565	0.864	0.348	0.6522	0.5158	0.2217	1.000	0.217	0.7826	0.7826
2.3737	0.773	0.391	0.6087	0.3814	1.1648	0.818	0.348	0.6522	0.4704	0.2276	1.000	0.174	0.8261	0.8261
2.3826	0.773	0.348	0.6522	0.4249	1.1824	0.773	0.348	0.6522	0.4249	0.2444	1.000	0.130	0.8696	0.8696
2.3867	0.727	0.348	0.6522	0.3794	1.1970	0.773	0.304	0.6957	0.4684	0.2726	0.955	0.130	0.8696	0.8241
2.3968	0.727	0.304	0.6957	0.4229	1.2264	0.773	0.261	0.7391	0.5119	0.4155	0.909	0.130	0.8696	0.7787
2.4082	0.727	0.261	0.7391	0.4664	1.2577	0.773	0.217	0.7826	0.5553	0.5483	0.864	0.130	0.8696	0.7332
2.4229	0.727	0.217	0.7826	0.5099	1.2668	0.773	0.174	0.8261	0.5988	0.5923	0.818	0.130	0.8696	0.6877
2.4331	0.727	0.174	0.8261	0.5534	1.2751	0.727	0.174	0.8261	0.5534	0.6534	0.818	0.087	0.9130	0.7312
2.4438	0.682	0.174	0.8261	0.5079	1.2896	0.682	0.174	0.8261	0.5079	0.7179	0.773	0.087	0.9130	0.6858
2.4579	0.682	0.130	0.8696	0.5514	1.3018	0.682	0.130	0.8696	0.5514	0.7604	0.727	0.087	0.9130	0.6403
2.4705	0.636	0.130	0.8696	0.5059	1.3138	0.682	0.087	0.9130	0.5949	0.8047	0.727	0.043	0.9565	0.6838
2.4936	0.636	0.087	0.9130	0.5494	1.3391	0.636	0.087	0.9130	0.5494	0.8682	0.682	0.043	0.9565	0.6383
2.5442	0.636	0.043	0.9565	0.5929	1.3942	0.591	0.087	0.9130	0.5040	0.8896	0.636	0.043	0.9565	0.5929
2.5991	0.591	0.043	0.9565	0.5474	1.4409	0.545	0.087	0.9130	0.4585	0.8921	0.636	0.000	1.0000	0.6364
2.6264	0.545	0.043	0.9565	0.5020	1.4478	0.500	0.087	0.9130	0.4130	0.9129	0.591	0.000	1.0000	0.5909
2.6446	0.545	0.000	1.0000	0.5455	1.4499	0.455	0.087	0.9130	0.3676	0.9376	0.545	0.000	1.0000	0.5455
2.6702	0.500	0.000	1.0000	0.5000	1.4599	0.409	0.087	0.9130	0.3221	0.9609	0.500	0.000	1.0000	0.5000
2.6952	0.455	0.000	1.0000	0.4545	1.4866	0.409	0.043	0.9565	0.3656	0.9818	0.455	0.000	1.0000	0.4545
2.7119	0.409	0.000	1.0000	0.4091	1.5053	0.364	0.043	0.9565	0.3202	0.9847	0.409	0.000	1.0000	0.4091
2.7213	0.364	0.000	1.0000	0.3636	1.5330	0.318	0.043	0.9565	0.2747	0.9859	0.364	0.000	1.0000	0.3636
2.7236	0.318	0.000	1.0000	0.3182	1.5655	0.273	0.043	0.9565	0.2292	0.9874	0.318	0.000	1.0000	0.3182
2.7602	0.273	0.000	1.0000	0.2727	1.5834	0.273	0.000	1.0000	0.2727	0.9891	0.273	0.000	1.0000	0.2727
2.8261	0.227	0.000	1.0000	0.2273	1.5994	0.227	0.000	1.0000	0.2273	0.9924	0.227	0.000	1.0000	0.2273
2.9071	0.182	0.000	1.0000	0.1818	1.6759	0.182	0.000	1.0000	0.1818	0.9964	0.182	0.000	1.0000	0.1818
2.9701	0.136	0.000	1.0000	0.1364	1.7594	0.136	0.000	1.0000	0.1364	0.9985	0.136	0.000	1.0000	0.1364
3.0024	0.091	0.000	1.0000	0.0909	1.7936	0.091	0.000	1.0000	0.0909	0.9987	0.091	0.000	1.0000	0.0909
3.0245	0.045	0.000	1.0000	0.0455	1.8686	0.045	0.000	1.0000	0.0455	0.9993	0.045	0.000	1.0000	0.0455
4.0261	0.000	0.000	1.0000	0.0000	2.9200	0.000	0.000	1.0000	0.0000	1.0000	0.000	0.000	1.0000	0.0000

Supplementary Figure 2. ROC curve for the combination of serum C1q and type-1 IFN signature for the prediction of active TB disease.

Supplementary Table 1.

Formula used to calculate type-1 IFN signature score:

$$\sum(Log\Delta Ct(subjects) - \overline{Log\Delta Ct(healthy\ controls)}) * st.dev.\ Log\Delta Ct(healthy\ controls)^{-1}$$

Genes	Genes	Genes	Genes
Tuberculosis	Primary Sjögren's Syndrome	Systemic Lupus Erythematosus	Systemic Sclerosis
UBE2L6	IFI44	IFI44	IFI44
FCGR1B	IFI44L	IFI44L	IFI44L
GBP1	IFIT1	IFIT1	IFIT1
IL1B	IFIT3	IFIT3	IFIT3
MYD88	MxA	MxA	MxA
TLR8			
IRF7			
STAT1			
SERPING1			
IFIT2			

Supplementary Table 2. ROC curve coordinates. Receiver operating characteristics (ROC) curve coordinates and Youden's index calculation indicating performance of serum C1q levels to distinguish APTB patients (cohort 1 + 2; n = 72) from healthy controls (cohort 1 + 2; n = 73). Statistical analysis was performed on the transformed dataset.

Coordinates of the Curve									
Positive if Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J	Positive if Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J
1,1086	1,0000	1,0000	0,0000	0,0000	2,4094	0,6944	0,3014	0,6986	0,3931
2,1101	1,0000	0,9863	0,0137	0,0137	2,4099	0,6944	0,2877	0,7123	0,4068
2,1357	1,0000	0,9726	0,0274	0,0274	2,4103	0,6806	0,2877	0,7123	0,3929
2,1627	1,0000	0,9589	0,0411	0,0411	2,4118	0,6667	0,2877	0,7123	0,3790
2,1658	0,9861	0,9589	0,0411	0,0272	2,4136	0,6667	0,2740	0,7260	0,3927
2,1748	0,9861	0,9452	0,0548	0,0409	2,4146	0,6528	0,2740	0,7260	0,3788
2,1840	0,9861	0,9315	0,0685	0,0546	2,4161	0,6528	0,2603	0,7397	0,3925
2,1876	0,9861	0,9178	0,0822	0,0683	2,4171	0,6528	0,2466	0,7534	0,4062
2,1917	0,9861	0,9041	0,0959	0,0820	2,4245	0,6528	0,2329	0,7671	0,4199
2,2030	0,9861	0,8904	0,1096	0,0957	2,4322	0,6528	0,2192	0,7808	0,4336
2,2163	0,9861	0,8767	0,1233	0,1094	2,4331	0,6528	0,2055	0,7945	0,4473
2,2198	0,9861	0,8630	0,1370	0,1231	2,4383	0,6389	0,2055	0,7945	0,4334
2,2213	0,9722	0,8630	0,1370	0,1092	2,4457	0,6389	0,1918	0,8082	0,4471
2,2221	0,9722	0,8493	0,1507	0,1229	2,4487	0,6250	0,1918	0,8082	0,4332
2,2226	0,9722	0,8356	0,1644	0,1366	2,4515	0,6111	0,1918	0,8082	0,4193
2,2231	0,9722	0,8219	0,1781	0,1503	2,4577	0,6111	0,1781	0,8219	0,4330
2,2244	0,9722	0,8082	0,1918	0,1640	2,4617	0,5972	0,1781	0,8219	0,4191
2,2275	0,9722	0,7945	0,2055	0,1777	2,4624	0,5833	0,1781	0,8219	0,4053
2,2334	0,9583	0,7945	0,2055	0,1638	2,4654	0,5694	0,1781	0,8219	0,3914
2,2399	0,9444	0,7945	0,2055	0,1499	2,4682	0,5556	0,1781	0,8219	0,3775
2,2502	0,9444	0,7808	0,2192	0,1636	2,4685	0,5417	0,1781	0,8219	0,3636
2,2583	0,9444	0,7671	0,2329	0,1773	2,4696	0,5417	0,1644	0,8356	0,3773
2,2594	0,9306	0,7671	0,2329	0,1634	2,4712	0,5417	0,1507	0,8493	0,3910
2,2624	0,9306	0,7534	0,2466	0,1771	2,4721	0,5417	0,1370	0,8630	0,4047
2,2660	0,9306	0,7397	0,2603	0,1908	2,4739	0,5417	0,1233	0,8767	0,4184
2,2681	0,9167	0,7397	0,2603	0,1769	2,4772	0,5278	0,1233	0,8767	0,4045
2,2706	0,9167	0,7260	0,2740	0,1906	2,4790	0,5278	0,1096	0,8904	0,4182
2,2724	0,9167	0,7123	0,2877	0,2043	2,4812	0,5278	0,0959	0,9041	0,4319
2,2739	0,9028	0,7123	0,2877	0,1904	2,4835	0,5278	0,0822	0,9178	0,4456

Supplementary Table 2 - continued

Coordinates of the Curve									
Positive if Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J	Positive if Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J
2,2769	0,9028	0,6986	0,3014	0,2041	2,4869	0,5139	0,0822	0,9178	0,4317
2,2813	0,9028	0,6849	0,3151	0,2178	2,4967	0,5000	0,0822	0,9178	0,4178
2,2857	0,9028	0,6712	0,3288	0,2315	2,5038	0,5000	0,0548	0,9452	0,4452
2,2885	0,9028	0,6575	0,3425	0,2452	2,5048	0,4861	0,0548	0,9452	0,4313
2,2930	0,9028	0,6438	0,3562	0,2589	2,5068	0,4722	0,0548	0,9452	0,4174
2,2985	0,9028	0,6301	0,3699	0,2726	2,5150	0,4722	0,0411	0,9589	0,4311
2,3017	0,9028	0,6164	0,3836	0,2863	2,5230	0,4583	0,0411	0,9589	0,4172
2,3041	0,9028	0,6027	0,3973	0,3000	2,5269	0,4444	0,0411	0,9589	0,4033
2,3061	0,9028	0,5890	0,4110	0,3137	2,5354	0,4306	0,0411	0,9589	0,3895
2,3076	0,8889	0,5890	0,4110	0,2998	2,5438	0,4167	0,0411	0,9589	0,3756
2,3101	0,8889	0,5753	0,4247	0,3135	2,5480	0,4028	0,0411	0,9589	0,3617
2,3130	0,8750	0,5753	0,4247	0,2997	2,5589	0,3889	0,0411	0,9589	0,3478
2,3178	0,8611	0,5753	0,4247	0,2858	2,5700	0,3750	0,0411	0,9589	0,3339
2,3241	0,8611	0,5616	0,4384	0,2995	2,5760	0,3611	0,0411	0,9589	0,3200
2,3301	0,8611	0,5479	0,4521	0,3132	2,5823	0,3472	0,0411	0,9589	0,3061
2,3341	0,8611	0,5342	0,4658	0,3269	2,5860	0,3333	0,0411	0,9589	0,2922
2,3349	0,8472	0,5342	0,4658	0,3130	2,5910	0,3194	0,0411	0,9589	0,2783
2,3393	0,8333	0,5342	0,4658	0,2991	2,5957	0,3194	0,0274	0,9726	0,2920
2,3437	0,8333	0,5205	0,4795	0,3128	2,6025	0,3194	0,0137	0,9863	0,3057
2,3452	0,8333	0,5068	0,4932	0,3265	2,6087	0,3056	0,0137	0,9863	0,2919
2,3465	0,8333	0,4932	0,5068	0,3402	2,6137	0,2917	0,0137	0,9863	0,2780
2,3492	0,8194	0,4932	0,5068	0,3263	2,6264	0,2778	0,0137	0,9863	0,2641
2,3523	0,8194	0,4795	0,5205	0,3400	2,6360	0,2778	0,0000	1,0000	0,2778
2,3541	0,8194	0,4658	0,5342	0,3537	2,6407	0,2639	0,0000	1,0000	0,2639
2,3557	0,8194	0,4521	0,5479	0,3674	2,6481	0,2500	0,0000	1,0000	0,2500
2,3565	0,8194	0,4384	0,5616	0,3811	2,6530	0,2361	0,0000	1,0000	0,2361
2,3580	0,8056	0,4384	0,5616	0,3672	2,6667	0,2222	0,0000	1,0000	0,2222
2,3617	0,8056	0,4247	0,5753	0,3809	2,6827	0,2083	0,0000	1,0000	0,2083
2,3647	0,7917	0,4247	0,5753	0,3670	2,6908	0,1944	0,0000	1,0000	0,1944
2,3679	0,7917	0,4110	0,5890	0,3807	2,6999	0,1806	0,0000	1,0000	0,1806
2,3710	0,7917	0,3973	0,6027	0,3944	2,7119	0,1667	0,0000	1,0000	0,1667

Supplementary Table 2 - continued

Coordinates of the Curve									
Positive if Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J	Positive if Greater Than or Equal To	Sensitivity	1-Specificity	Specificity	Youden index J
2,3732	0,7778	0,3973	0,6027	0,3805	2,7213	0,1528	0,0000	1,0000	0,1528
2,3784	0,7639	0,3973	0,6027	0,3666	2,7236	0,1389	0,0000	1,0000	0,1389
2,3819	0,7639	0,3836	0,6164	0,3803	2,7359	0,1250	0,0000	1,0000	0,1250
2,3826	0,7639	0,3699	0,6301	0,3940	2,7546	0,1111	0,0000	1,0000	0,1111
2,3857	0,7500	0,3699	0,6301	0,3801	2,7723	0,0972	0,0000	1,0000	0,0972
2,3885	0,7500	0,3562	0,6438	0,3938	2,7896	0,0833	0,0000	1,0000	0,0833
2,3895	0,7361	0,3562	0,6438	0,3799	2,8261	0,0694	0,0000	1,0000	0,0694
2,3920	0,7361	0,3425	0,6575	0,3936	2,9071	0,0556	0,0000	1,0000	0,0556
2,3949	0,7361	0,3288	0,6712	0,4073	2,9701	0,0417	0,0000	1,0000	0,0417
2,3997	0,7361	0,3151	0,6849	0,4210	3,0024	0,0278	0,0000	1,0000	0,0278
2,4059	0,7361	0,3014	0,6986	0,4347	3,0245	0,0139	0,0000	1,0000	0,0139
2,4085	0,7222	0,3014	0,6986	0,4209	4,0261	0,0000	0,0000	1,0000	0,0000
2,4088	0,7083	0,3014	0,6986	0,4070					

CHAPTER 4

Circulating levels of anti-C1q and anti-factor H autoantibodies and their targets in normal pregnancy and preeclampsia

Douwe J. Dijkstra[1], A. Inkeri Lokki[2,3], Lobke M. Gierman[4], Nicole V. Borggreven[1], Carin van der Keur[1], Michael Eikmans[1], Kyra A. Gelderman[5], Hannele Laivuori[3,6,7], The FINNPEC Core Investigator Group, Ann-Charlotte Iversen[4], Marie-Louise P. van der Hoorn[8], Leendert A. Trouw[1]

1. Department of Immunology, Leiden University Medical Center, Leiden, the Netherlands
2. Department of Bacteriology and Immunology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
3. Medical and Clinical Genetics, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
4. Centre of Molecular Inflammation Research, Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway
5. Department of Immunopathology and Haemostasis, Sanquin Diagnostic Services, Amsterdam, the Netherlands
6. Institute for Molecular Medicine Finland, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland
7. Department of Obstetrics and Gynecology, Tampere University Hospital and Tampere University, Faculty of Medicine and Health Technology, Tampere Center for Child, Adolescent, and Maternal Health Research, Tampere, Finland
8. Department of Obstetrics and Gynecology, Leiden University Medical Center, Leiden, the Netherlands

Frontiers in Immunology (2022), doi: *10.3389/fimmu.2022.842451*

Abstract

Preeclampsia (PE) generally manifests in the second half of pregnancy with hypertension and proteinuria. The understanding of the origin and mechanism behind PE is incomplete, although there is clearly an immune component to this disorder. The placenta constitutes a complicated immune interface between fetal and maternal cells, where regulation and tolerance are key. Stress factors from placental dysfunction in PE are released to the maternal circulation evoking the maternal response. Several complement factors play a role within this intricate landscape, including C1q in vascular remodeling and Factor H (FH) as the key regulator of alternative pathway complement activation. We hypothesize that decreased levels of C1q or FH, or disturbance of their function by autoantibodies, may be associated with PE. Autoantibodies against C1q and FH and the concentrations of C1q and FH were measured by ELISA in maternal sera from women with preeclamptic and normal pregnancies. Samples originated from cohorts collected in the Netherlands (n = 63 PE; n = 174 control pregnancies, n = 51 nonpregnant), Finland (n = 181 PE; n = 63 control pregnancies) and Norway (n = 59 PE; n = 27 control pregnancies). Serum C1q and FH concentrations were higher in control pregnancy than in nonpregnant women. No significant differences were observed for serum C1q between preeclamptic and control pregnancy in any of the three cohorts. Serum levels of FH were lower in preeclamptic pregnancies compared to control pregnancies in two of the cohorts, this effect was driven by the early onset PE cases. Neither anti-C1q autoantibodies nor anti-FH autoantibodies levels differed between women with PE and normal pregnancies. In conclusion, levels of anti-C1q and anti-FH autoantibodies are not increased in PE. C1q and FH are increased in pregnancy, but importantly, a decrease in FH concentration is associated with PE.

Introduction

Preeclampsia (PE) is a vascular complication presenting in the second half of pregnancy. PE occurs in about 3% of pregnancies and is an important cause of both fetal and maternal morbidity [1]. The disease is characterized by newly developed hypertension and proteinuria, or new-onset PE-associated signs in the absence of proteinuria, and can progress systemically to include organ dysfunction. Onset of PE is unpredictable, with the only cure being delivery of the placenta [2]. Although the mechanism behind the development of PE is incompletely understood, its origin may involve improper placentation with incomplete remodeling of the uterine spiral arteries and development of a dysfunctional placenta with vascular malperfusion [3]. As the pregnancy progresses and the fetus demands a fully functional placenta, the placenta releases increasing amounts of stress signals to the maternal circulation and the mother may eventually develop endothelial dysfunction and the clinical manifestation of PE.

In the context of healthy pregnancy, a well-functioning and regulated complement system is key. The complement system is an important component of the innate immune system, consisting of more than 30 protein factors that form three converging activation pathways (classical, lectin and alternative pathways) with their accessory regulators [4]. Although classically known in the defense against infections, the complement system is also involved in clearance of immune complexes and dying cells, tissue regeneration and angiogenesis [5]. Additionally, complement forms a bridge towards cellular immune responses, attracting and activating immune cells locally with activation fragments that act as anaphylatoxins and opsonins.

The initiator of the classical pathway of the complement system, C1q, binds directly to apoptotic cells and debris that results from tissue remodeling, labeling it for clearance [6]. The relevance of C1q for a healthy pregnancy was highlighted by the observation of impaired labyrinth development and vessel remodeling in pregnant C1q-deficient mice compared to wildtype mice [7]. Additionally, C1q-deficient mice are predisposed to present with PE-like symptoms and higher fetal loss [8]. In human pregnancy, C1q is found throughout the maternal side of the placenta and is also locally produced by invading trophoblasts, the fetal cell type directly interacting with maternal cells at the maternal-fetal interface [7]. In healthy pregnancy the local presence of C1q in the placenta does not result in overt complement activation [9]. Human C1q deficiency is very rare and although it is studied in great detail because of its association with development of Systemic Lupus Erythematosus (SLE) and infections, no information is currently available on pregnancy outcomes [10].

Factor H (FH) is a key regulator of the alternative pathway because of its ability to block C3 convertase formation and its function as a cofactor for Factor I. In this way, FH plays an important role in the steady state protection of host cells throughout the body, especially circulating cells and endothelial cells. In addition, FH provides protection against excessive complement activation on apoptotic cells, which downregulate membrane-bound complement inhibitors [11]. FH is abundantly present in the placenta, but the observed irregular distribution of FH in the PE placenta may suggest a disturbed balance between complement activation and regulation in PE [12]. FH was even observed intracellularly in syncytiotrophoblast, although this FH may actually be of fetal rather than maternal origin [13].

Autoantibodies may target multiple complement proteins, including C1q and FH [14]. Anti-C1q autoantibodies are typically present in diseases like SLE, Hypocomplementemic Urticarial Vasculitis Syndrome and Rheumatoid Vasculitis, but are also present in a fraction of healthy individuals [15]. Importantly, anti-C1q autoantibodies do not deplete circulating C1q, but may amplify classical pathway driven complement activation and dysregulate other processes driven by C1q [16, 17]. Autoantibodies to FH have been described in atypical Hemolytic Uremic Syndrome and C3-glomerulopathy as well as in antiphospholipid syndrome and other autoimmune diseases [18, 19]. Anti-FH autoantibodies may have different functional consequences, as some form immune complexes leading to (partial) FH depletion, while other anti-FH autoantibodies affect the functional properties of FH [19]. Consequences of these autoantibodies are often associated with autoimmune diseases, where they may drive or enhance pathogenesis. Several previous studies showed a relationship between anti-C1q autoantibodies and negative pregnancy outcomes in miscarriage, ectopic pregnancy and autoimmune thyroid disorders, but not for PE [20-22]. A study focusing on pregnancy in lupus nephritis patients found that anti-C1q was not a predictor of PE [23].

We hypothesize that a balance between complement activation, needed for tissue remodeling and clearance of apoptotic cells in the placenta, and regulation is critical for a healthy pregnancy. This balance may be disturbed in PE, which could contribute to the underlying pathogenesis. Autoantibodies against complement factors may further hamper their proper function or incite unwanted immune activation. In this study, we therefore compared the serum levels of C1q and FH, and autoantibodies against these factors, in women with healthy or preeclamptic pregnancies.

Materials and methods

Patients and samples

Serum samples of women with preeclamptic and control pregnancies were derived from three cohorts collected in the Netherlands, Finland and Norway. PE was defined as hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg) and proteinuria (≥0.3 g/24h or ≥0.3 g/L), or newly developed PE-associated signs in the absence of proteinuria, with onset beyond 20 weeks of gestation. Women with normal pregnancies and no previous history of PE were included as controls.

The cohort from the Netherlands was collected at the Leiden University Medical Center (LUMC) and comprised 63 women with PE and 174 women with control pregnancies. Serum samples from these women were collected on the day of delivery, although it varied whether this was performed before or after the actual delivery. Additionally, a group of 51 nonpregnant women with a similar age profile as the pregnancy cohorts (median age 31 years, range 20-46), was included.

The cohort from Finland comprises samples of 181 preeclamptic and 63 control pregnancies from the Finnish Genetics of Pre-eclampsia Consortium (FINNPEC) that has been described before [24]. In this cohort proteinuria could additionally be diagnosed based on two ≥1 readings on a dipstick in a random urine determination with no evidence of a urinary tract infection. Samples were collected at recruitment, 74% were taken prior to the day of delivery (range 1 to 31 days before delivery), while 25% of samples were taken on the day of delivery, one sample in the control pregnancy group was taken on the second day after delivery.

The Norwegian cohort was collected at St. Olavs and Haukeland University Hospitals and comprises sera from 59 women with PE and 27 women with control pregnancies. All samples in this cohort were collected before delivery on the day of delivery by caesarean section, without signs of being in labor. Caesarean sections for the control group were indicated due to breech position, suspected birth defects, previous obstetric history, or birth anxiety. Serum samples from Finland and Norway were stored frozen and were transported to LUMC for analysis. Ethical approval for the three cohorts was obtained at the individual centers (the Netherlands: P08.229/228, Finland: 149/EO/2007, Norway: REC 2012/1040).

Anti-C1q antibody ELISA

Antibodies against C1q were measured by QUANTA Lite Anti-C1q ELISA (Inova Diagnostics) according to the manufacturer's protocol. Briefly, 100 µl of 1:101 diluted

sera in Samples Diluent were incubated in ELISA plate wells for 30 minutes at room temperature. Wells were washed 3 times with wash buffer, incubated with 100 µl horseradish peroxidase (HRP) conjugated anti-IgG and incubated for 30 minutes at room temperature. After washing 3 times, wells were incubated with 100 µl TMB Chromogen for 30 minutes at room temperature, followed by addition of 100 µl acidic HRP Stop Solution. Absorbance values were read at 450 nm and used to calculate anti-C1q units based positive control samples included in the kit. The cut-off for positivity was 20 units, as recommended by the manufacturer.

Anti-FH antibody ELISA

Measurement of antibodies against FH was performed using an in-house developed ELISA. Nunc MaxiSorp ELISA plates (ThermoScientific) were coated with 50 µl of 10 µg/ml FH (Complement Technology) in 0.1M bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Wells were washed 3 times with PBS/0.05% Tween, blocked with 100 µl PBS/1% BSA for 1 hour at 37°C and washed 3 times. Serum samples were diluted 1:50 in PBS/0.05% Tween/1% BSA (PTB) and 50 µl sample was incubated in wells for 1 hour at 37°C. After washing 3 times, 50 µl 0.11 µg/ml Goat anti-human IgG-biotin (Invitrogen) was added, followed by incubation for 1 hour at 37°C and washing 3 times. Secondary detection consisted of 50 µl 0.5 µg/ml streptavidin-HRP (ThermoScientific), incubated for 1 hour at 37°C. After the final washing sequence, 50 µl 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)/0.015% H_2O_2 was added. Absorbance was read at 415 nm. Anti-FH units (U) were determined based on standard curve of anti-FH standard (180kU; Sanquin Diagnostics, Amsterdam, the Netherlands) by an Excel-based logit calculation. Cut-off for positivity was set at 95th percentile of all control pregnancy samples combined.

C1q ELISA

To measure the concentration of C1q in serum samples, an in-house ELISA was performed as described before [25]. Nunc MaxiSorp ELISA plates (ThermoScientific) were coated with 50 µl 2.5 µg/ml mouse anti-human C1q monoclonal antibody (mAb) 2204 (kind gift Prof. C. van Kooten, Dept Nephrology, LUMC) in 0.1M bicarbonate coating buffer (pH 9.6) and incubated overnight at room temperature. Wells were washed 3 times with PBS/0.05% Tween and blocked with 100 µl PBS/1% BSA and incubated for 1 hour at 37°C. After washing, 50 µl of serially diluted serum samples in PTB, as well as a standard curve from a pool of normal human serum were added to the wells, followed by incubation for 1 hour at 37°C and washing 3 times. Wells were then incubated with 50

µl 1:2000 diluted rabbit anti-human C1q (DAKO) for 1 hour at 37°C. After washing, 50 µl 1:2000 goat anti-rabbit-HRP (DAKO) was added for detection and wells were incubated for 1 hour at 37°C. Following the final washing sequence, wells were incubated with 50 µl ABTS/0.015% H_2O_2 and absorbance was read at 415 nm. C1q concentrations were calculated based on the standard curve of a reference serum.

FH ELISA

Concentration of FH in serum was determined by an in-house ELISA on Nunc MaxiSorp ELISA plates (ThermoScientific). Wells were coated with 50 µl 0.5 µg/ml mouse anti-FH (clone FH.16; Sanquin) in bicarbonate coating buffer (pH 9.6) and incubated overnight at room temperature. After washing 3 times with PBS/0.05% Tween, wells were blocked with 100 µl PBS/1% BSA for 1 hour at 37°C. Samples were diluted 1:2000 and 1:4000 in PTB, and 50 µl was added to the wells. For calculation of FH concentration, a standard curve of a reference serum with known FH concentration was added on each plate. Wells were incubated for 1 hour at 37°C and washed, then incubated with 50 µl 0.25 µg/ml biotinylated mouse anti-human FH mAb OX-23 for 1 hour at 37°C. After washing, detection was performed by incubating the wells with 50 µl 0.5 µg/ml streptavidin-HRP (ThermoScientific) for 1 hour at 37°C. Following the final wash, wells were stained with 50 µl ABTS/0.015% H_2O_2 and absorbance was read at 415 nm. FH concentrations were calculated based on the standard curve of a reference serum.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA, USA). Normal distribution of data was examined by D'Agostino & Pearson normality test. Comparisons of numerical data were performed by Mann-Whitney U test, whereas categorical data was analyzed with Fisher's exact test. Analysis of samples matched for gestational age (in the cohort from Finland) was performed by forming pairs with maximum 2 days difference in gestational age, C1q and Factor H concentrations were then compared between pairs by paired t test. Correlation between data was analyzed by Spearman's rank correlation.

Table 1. Clinical characteristics of the included subjects. For patient characteristics, median and range are indicated. For blood pressure, the highest diastolic blood pressure is used. Where information was not available for all subjects, the number of missing data points is indicated in superscript. Statistical significance calculated by Mann Whitney U test (#) or Fisher's exact test ($).

	The Netherlands		
	Preeclampsia (n = 63)	**Control (n = 174)**	**p-value**
Maternal age (years)	31 (18-46)	33 (23-42)	**0.0117**#
Gestational age (days)	220 (170-283)	275 (266-297)	**<0.0001**#
Fetal birthweight (g)	1246 (380-4030)	3598 (2445-5100)	**<0.0001**#
Blood pressure (mmHg)	104 (90-160)	75 (55-90)	**<0.0001**#
Proteinuria (mg/24hr)	2300 (310-14000) [16]	N/A	N/A
Gravidity	2 (1-9)	3 (1-9)	**<0.0001**#
Parity	0 (0-5)	1 (0-6)	**<0.0001**#
Caesarean section (%)	49 (78%)	113 (65%)	0.0814$
	Finland		
	Preeclampsia (n = 181)	**Control (n = 63)**	**p-value**
Maternal age (years)	32 (18-47)	31 (21-43)	**<0.0001**#
Gestational age (days)	246 (165-286) [1]	280 (167-297) [1]	**<0.0001**#
Fetal birthweight (g)	2775 (310-5110)	3470 (330-4748)	**<0.0001**#
Blood pressure (mmHg)	111 (91-173)	84 (67-130) [1]	**<0.0001**#
Proteinuria (mg/L)	3200 (300-19100) [3]	200-270 [60]	**<0.0001**#
Gravidity	1 (1-18)	2 (1-5) [1]	**<0.0001**#
Parity	0 (0-12)	1 (1-2) [1]	0.4621#
Caesarean section (%)	90 (50%)	25 (40%) [1]	0.2389$
BMI (start of pregnancy)	23.90 (18.20-48.40)	22.90 (18.20-39.10) [1]	**<0.0001**#
	Norway		
	Preeclampsia (n = 45)	**Control (n = 27)**	**p-value**
Maternal age (years)	30 (20-45)	33 (23-41)	**0.0447**#
Gestational age (days)	223 (178-275)	274 (265-294)	**<0.0001**#
Fetal birthweight (g)	1286 (550-5010)	3510 (2800-4330)	**<0.0001**#
Blood pressure (mmHg)	100 (80-130)	75 (60-99)	**<0.0001**#
Proteinuria (Yes / No)	Yes: 45	No: 26 [1]	**<0.0001**$
Gravidity	2 (1-13)	2 (1-5)	0.0743#
Parity	0 (0-3)	1 (0-3)	**0.0095**#
Caesarean section (%)	45 (100%)	27 (100%)	>0.9999$
BMI (start of pregnancy)	24.4 (18.2-38.4) [4]	24.6 (16.4-33.8) [2]	0.522

Results

Patient characteristics

To study the relationship between the presence of anti-complement autoantibodies and complement protein levels and PE we analyzed serum samples obtained from cohorts from the Netherlands, Finland and Norway, comprising a total of 289 preeclamptic pregnancies and 264 control pregnancies. Three cohorts from different countries were investigated to provide a robust basis for conclusions. Overall, the three cohorts displayed the expected clinical characteristics associated with PE, such as increased diastolic blood pressure,

proteinuria and lower gestational age in the PE-complicated pregnancies as compared to the control pregnancies (Table 1). Both gravidity and parity were in general lower in the PE group than in controls although some differences existed between the cohorts. The proportion of deliveries performed through Caesarean section was not significantly different between the groups in any of the cohorts. Maternal body mass index (BMI) was higher in the Finnish PE group as compared to the control group, no difference in BMI was present in Norway, while BMI data from the Netherlands was not available. When comparing the three cohorts of PE cases, the cohort from Finland stands out with a higher median gestational age and fetal birthweight, (Table 2). Moreover, among both PE cases and controls, a lower frequency of Caesarean sections is present in the Finnish cohort.

Table 2. Comparison of clinical characteristics between the three independent cohorts in control pregnancies and in PE cases. Where information was not available for all subjects, the number of missing data points is indicated in superscript. All comparisons were performed with Kruskal-Wallis test, except for the mode of delivery, which was analyzed by Chi-square test.

Control pregnancies				
Parameter	**The Netherlands (n = 174)**	**Finland (n = 63)**	**Norway (n = 27)**	**p-value**
Maternal age (years)	33 (23-42)	31 (21-43)	33 (23-41)	0.032
Gestational age (days)	275 (266-297)	280 (167-297)[(1)]	274 (265-294)	0.205
Fetal birthweight (g)	3598 (2445-5100)	3470 (330-4748)	3510 (2800-4330)	0.089
Blood pressure (mmHg)	75 (55-90)	84 (67-130)[(1)]	75 (60-99)	**<0.0001**
Gravidity	3 (1-9)	2 (1-5)[(1)]	2 (1-5)	**0.0006**
Parity	1 (0-6)	1 (1-2)[(1)]	1 (0-3)	**<0.0001**
Caesarean section (%)	113 (65%)	25 (40%)[(1)]	27 (100%)	**<0.0001**
Preeclampsia				
Parameter	**The Netherlands (n = 63)**	**Finland (n = 181)**	**Norway (n = 45)**	**p-value**
Maternal age (years)	31 (18-46)	32 (18-47)	30 (20-45)	0.409
Gestational age (days)	220 (170-283)	246 (165-286)[(1)]	223 (178-275)	**<0.0001**
Fetal birthweight (g)	1246 (380-4030)	2775 (310-5110)	1286 (550-5010)	**<0.0001**
Blood pressure (mmHg)	104 (90-160)	111 (91-173)	100 (80-130)	**<0.0001**
Gravidity	2 (1-9)	1 (1-18)	2 (1-13)	0.532
Parity	0 (0-5)	0 (0-12)	0 (0-3)	0.038
Caesarean section (%)	49 (78%)	90 (50%)	45 (100%)	**<0.0001**

Anti-C1q autoantibodies and C1q

Anti-C1q antibodies were absent in maternal serum of preeclamptic pregnancies in the cohort from the Netherlands, while 10.3% of control pregnancy sera and 17.6% of sera from nonpregnant women were positive for anti-C1q (Figure 1A). However, no significant differences between control and preeclamptic pregnancies were observed for anti-C1q levels in the other cohorts (Figures 1B, C, G). For serum C1q concentration, no significant differences were observed between the PE and control pregnancies in any

of the cohorts (Figures 1D–F). Analysis of pooled data from all cohorts and gestational age-matched samples confirmed the lack of association between C1q concentration and PE (Figures 1H, I). Interestingly, the serum C1q concentration was higher for both control and preeclamptic pregnancies than for nonpregnant women (Figure 1D).

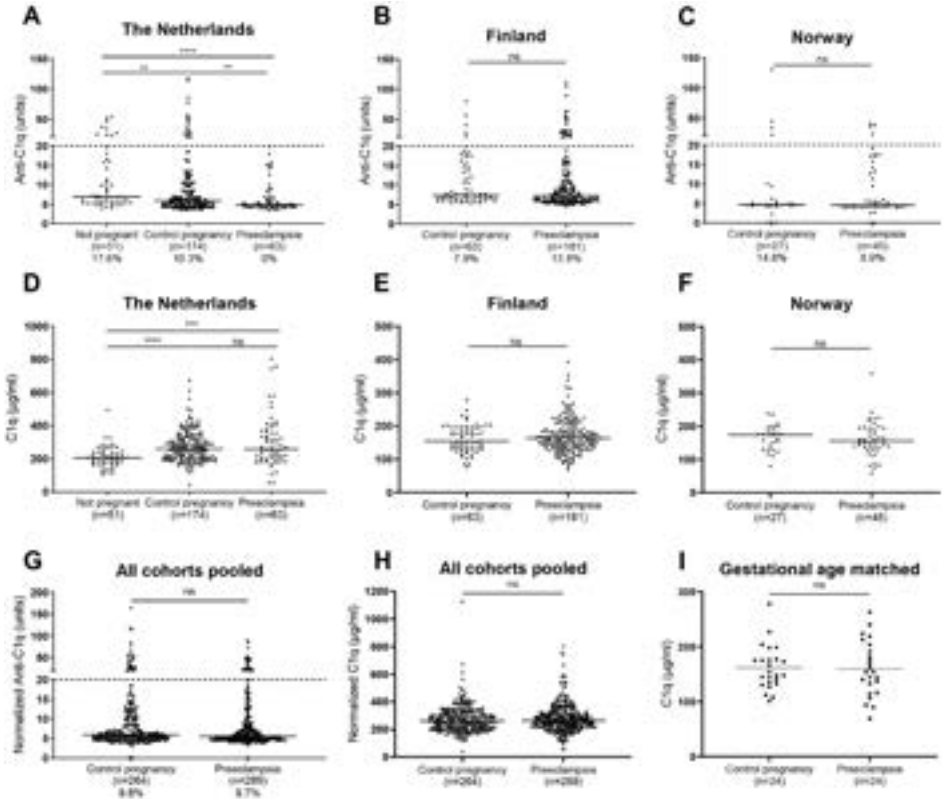


Figure 1. Anti-C1q antibodies and C1q in healthy or PE pregnancy. Anti-C1q antibodies measured in maternal serum in cohorts from the Netherlands (A), Finland (B) and Norway (C), with percentages under the graph indicating the proportion samples deemed anti-C1q positive (>20 units, also indicated by dashed line). C1q concentration measured in maternal serum in cohorts from the Netherlands (D), Finland (E) and Norway (F). Data from different cohorts was normalized and pooled for anti-C1q (G) and C1q (H). For the cohort from Finland, a subanalysis with samples matched for gestational age was additionally performed (I). Comparisons tested by Mann-Whitney, or paired t test for panel I; *, 0.05 < p < 0.01;**, 0.001 < p < 0.01; ***, 0.0001 < p < 0.001; ****, p < 0.0001.

Anti-FH autoantibodies and FH

We tested whether dysregulation of FH by the presence of autoantibodies is associated with PE, but no significant differences were observed regarding the levels of anti-FH autoantibodies between PE and control pregnancies (Figures 2A–C, G). However,

importantly we observed significantly decreased levels of FH comparing the preeclamptic pregnancies to healthy pregnancies. In the cohorts from the Netherlands and Norway, the FH concentration was significantly lower in the PE group compared to controls pregnancies (Figures 2D, F). In samples from Finland, a similar trend of lower FH concentration in the PE group was observed (p = 0.067) (Figure 2E). When pooled data from all cohorts were analyzed, the lower FH concentration remained significant (Figure 2H). Additionally, an analysis of samples matched for gestational age in the cohort from Finland shows the same result, excluding the possibility of data skewing by lower gestational ages in PE (Figure 2I). Similar to what was observed for C1q, women with control pregnancies showed higher FH concentration than nonpregnant controls (Figure 2D).

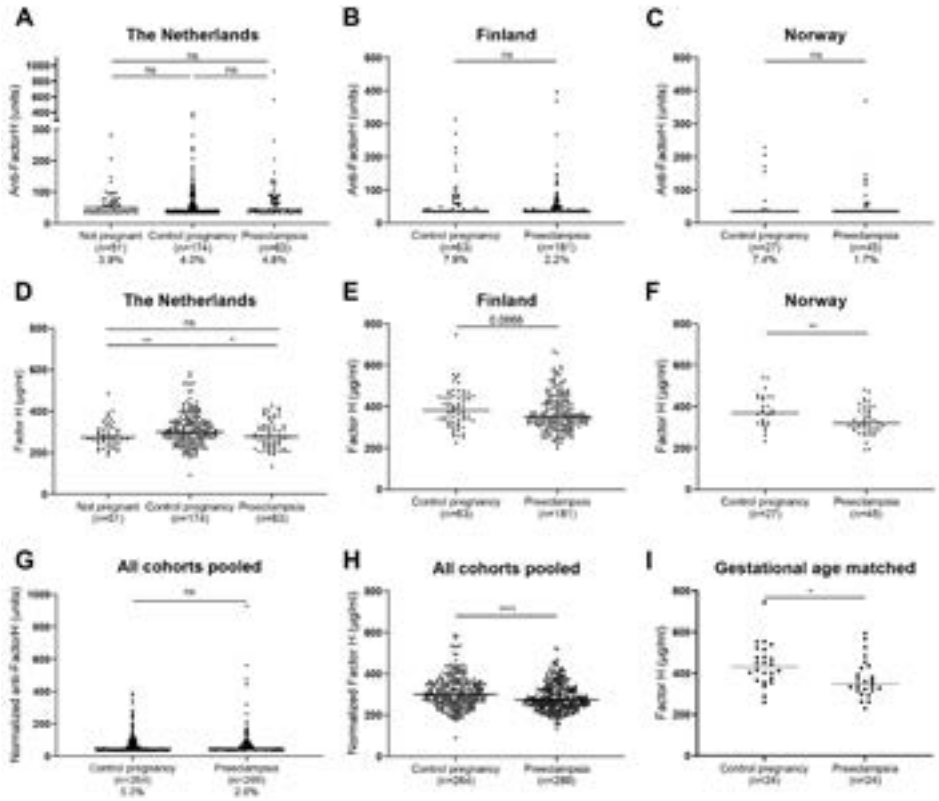


Figure 2. Anti-Factor H antibodies and Factor H in healthy or PE pregnancy. Anti-Factor H antibodies measured in maternal serum in cohorts from the Netherlands (A), Finland (B) and Norway (C), dotted line indicates the detection limit at 35.2 units. Factor H concentration measured in maternal serum in cohorts from the Netherlands (D), Finland (E) and Norway (F). Data from different cohorts was normalized and pooled for anti-FH (G) and FH (H). For the cohort from Finland, a subanalysis with samples matched for gestational age was additionally performed (I). Comparisons tested by Mann-Whitney, or paired t test for panel I; *, 0.05 < p < 0.01; **, 0.001 < p < 0.01; ***, 0.0001 < p < 0.001; ****, p < 0.0001.

Table 3. Correlation between clinical parameters and C1q, FH and autoantibodies in preeclamptic pregnancies.

The Netherlands (n = 63)								
Parameter	**Anti-C1q**		**C1q**		**Anti-FH**		**FH**	
	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value
Gestational age	-0.199	0.118	0.050	0.696	-0.248	0.050	0.023	0.861
Proteinuria	-0.243	0.100	-0.200	0.178	0.146	0.326	0.090	0.549
Highest diastole	0.078	0.541	-0.088	0.495	0.179	0.160	0.101	0.430
Fetal birthweight	-0.150	0.241	-0.074	0.566	**-0.291**	**0.021**	0.031	0.806
Finland (n = 181)								
Parameter	**Anti-C1q**		**C1q**		**Anti-FH**		**FH**	
	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value
Gestational age	-0.044	0.562	0.026	0.734	0.082	0.271	0.090	0.227
Proteinuria	-0.017	0.826	-0.069	0.360	0.008	0.911	**-0.157**	**0.037**
Highest diastole	-0.145	0.051	-0.073	0.330	**-0.198**	**0.007**	0.100	0.180
Fetal birthweight	-0.117	0.118	-0.023	0.756	0.005	0.948	0.135	0.071
BMI	-0.111	0.135	0.004	0.958	0.034	0.649	**0.288**	**8.57e-5**
Norway (n = 45)								
Parameter	**Anti-C1q**		**C1q**		**Anti-FH**		**FH**	
	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value
Gestational age	0.016	0.920	-0.125	0.412	0.042	0.785	**0.327**	**0.029**
Highest diastole	0.006	0.967	-0.155	0.311	-0.194	0.203	0.071	0.644
Fetal birthweight	0.061	0.691	-0.257	0.089	-0.025	0.873	0.250	0.098
BMI	0.025	0.877	0.306	0.052	0.008	0.961	**0.351**	**0.024**

Study of the association of complement and autoantibodies with clinical parameters and subgroups of PE patients

To further investigate the relationship between PE and C1q, FH or autoantibodies targeting these proteins, Spearman correlations with clinical parameters associated with PE were explored for all PE patients (Table 3). The clinical parameters included proteinuria and diastolic blood pressure, fetal birthweight and gestational age at delivery, (Table 1). Interestingly, a positive correlation was found between the mother's BMI and serum FH concentration in the cohorts from Finland and Norway (Spearman r = 0.288; p = 8.6e-5 and r = 0.351; p = 0.024 respectively). However, no other consistent significant correlations were found relating to PE symptoms or other relevant clinical parameters. Further analyses were performed on subgroups of PE patients based on time of disease onset or presence of fetal growth restriction (FGR) (Table 4). Early onset of PE (gestational age below 34 weeks) was associated with decreased FH concentration compared to late onset, this difference was significant in cohorts from Finland and Norway, while a similar trend was observed in the cohort from the Netherlands. PE with FGR was associated to higher serum levels of anti-FH antibodies compared

to preeclampsia with normal fetal growth, but this difference was only statistically significant in the cohort from the Netherlands.

Table 4. Analysis of complement factors C1q, FH and autoantibodies in subgroups based on early onset of disease or presence of fetal growth restriction. Medians of each groups are indicated, together with significance as determined with Mann-Whitney test. Early onset was defined as gestational age at delivery below 34 weeks; fetal growth restriction (FGR) was defined as gestational age-adjusted birthweight up to percentile 10 (of growth curve for respective country).

	The Netherlands		**Finland**		**Norway**	
	Early (n = 50) vs late (n = 13) onset PE	**p-value**	**Early (n = 36) vs late (n = 145) onset PE**	**p-value**	**Early (n = 33) vs late (n = 12) onset PE**	**p-value**
Anti-C1q	4.75 – 5.00	0.407	7.95 – 7.10	0.198	4.60 – 4.90	0.854
C1q	265 – 225	0.753	165 – 162.6	0.881	155 – 150	0.608
Anti-FH	56.7 – 37.3	0.122	35.2 – 35.2	0.194	35.2 – 35.2	0.392
FH	276 – 306	0.537	**322.5 – 354**	**0.019**	**306 – 381**	**0.001**
	The Netherlands		**Finland**		**Norway**	
	PE with (n = 17) vs without (n = 46) FGR	**p-value**	**PE with (n = 84) vs without (n = 97) FGR**	**p-value**	**PE with (n = 33) vs without (n = 12) FGR**	**p-value**
Anti-C1q	4.60 – 4.95	0.715	7.40 – 7.10	0.294	4.50 – 5.70	0.391
C1q	257 – 256	0.772	166.8 – 155.9	0.119	159 – 146	0.377
Anti-FH	**79.0 – 41.9**	**0.029**	35.2 – 35.2	0.369	35.2 – 35.2	0.253
FH	282 – 277	0.936	344.5 – 354	0.222	318 – 363	0.255

Discussion

In the current study, we investigated the relation between the pregnancy complication PE and the circulating levels of complement factors C1q and FH, as well as autoantibodies against these factors. We observed that the presence of autoantibodies against C1q or FH is not associated with PE. Circulating C1q and FH levels were higher in pregnant than in nonpregnant women. Decreased serum concentrations of FH were associated with PE, while this was not the case for serum C1q concentrations. Serum FH levels were lower in the subset of PE cases with earlier onset, compared to late onset cases.

Although the etiology of PE remains unclear, multiple immune mechanisms have been proposed to play a role, as the placenta constitutes a unique immune environment. Differentially expressed levels of fetal Human Leukocyte Antigen (HLA), especially HLA-G, immune cell influx and cytokine expression have been reported in preeclamptic placentas [26-30]. Complement has also been implicated in the development of PE. Our data now add to this understanding that the circulating levels of the endogenous complement inhibitor FH are decreased in PE. Several studies linked anti-C1q autoantibodies to various negative pregnancy outcomes, but not to PE [20-23]. The current study reinforces that the presence of anti-C1q does not associate with PE. Likewise, no evidence for a

link between anti-FH and PE was observed. These results contradict the hypothesis of a contribution by complement dysregulation by autoantibodies to development of PE.

C1q has multiple functions during pregnancy, e.g. in angiogenesis, tissue remodeling and clearance of cellular debris. These functions are supported by the ability of C1q to recognize exposed or altered self-structures, facilitating clearance without overt inflammation [31]. Next to observations in human pregnancy, data from experiments in mice indicate a key role for C1q in placentation as the litter size in C1q deficient mice is smaller as compared to wild-type mice [7]. The notion that C1q plays an important role in pregnancy fits with our finding of increased C1q levels in pregnant women, both complicated and control, as compared to nonpregnant women. Especially the role C1q plays in the clearance of apoptotic cells and debris is postulated to be important in the case of PE. Fragments of apoptotic syncytiotrophoblast from the placenta may enter the maternal circulation and the complement system assists in clearing these fragments by labelling them for phagocytosis. One previous study showed decreased C1q in severe PE cases, however this is contradicted by our finding that there was no difference in C1q levels between PE patients and controls [32].

In a prospective study increased FH and C1q levels were reported in maternal plasma of later PE patients relative to controls at 6 to 13 weeks of pregnancy [33]. However, these differences were not observed in the second and third trimesters. The current study investigated only third trimester samples and will therefore have missed differences in complement protein levels in the first trimester. However, the current study does find a difference in third trimester serum FH level, which was not observed in the previous study by He and colleagues.

The balance between complement activation and regulation is key in a healthy pregnancy, which is substantiated by findings that FH levels increase during pregnancy [32, 34]. This conclusion is corroborated by our results where women with control pregnancies had higher serum FH concentration than nonpregnant women. Abnormalities in activating and regulating components of the alternative complement pathway have been reported in PE. Multiple investigations found increased levels of activation fragment Bb in PE cases, indicating higher than normal alternative pathway activation [35, 36]. Another study found increased Bb as well as decreased FH levels in PE patients, although this study was limited by relatively small samples size and a focus on only severe PE cases [32]. Additionally, genetic variants in genes encoding the regulators Factor I and membrane cofactor protein, but not FH, were linked to PE [37]. This trend of disbalance of the alternative pathway in PE is supported by our finding that FH concentration is decreased

in PE patients. As the main fluid-phase complement regulator FH is conceivably of key importance in preventing or limiting C1q driven complement activation in the placenta. Aberrant FH levels or disturbed FH function would then result in the increased levels of complement activation as reported for PE.

Early onset PE cases showed lower FH concentration than late onset cases, implying that early onset cases were driving the lower FH concentration found in the general PE group compared to control pregnancies. This may also explain why FH concentration was not significantly lower in the cohort from Finland, as there were relatively fewer early onset cases in this cohort than in cohorts from the Netherlands and Norway. The larger variety in sampling timepoints in the Finnish cohort could also be involved. This study also investigated relations between the experimental data and relevant clinical parameters related to PE. No consistent significant correlations were found, indicating that within the PE pregnancies FH is not correlated with more severe disease. The observed correlation between BMI and FH is in line with earlier links found between FH and BMI [38, 39].

The strengths of the current analysis include the side-by-side comparison of three independent well-documented European cohorts, together comprising sizable numbers of cases and controls. In addition, all measurements were performed in the same lab with the same assays. Moreover, the current study also investigated correlations with several clinical parameters that could indicate severity of PE. The weaknesses of the study include the not completely identical sampling time points in the cohorts as samples in the Netherlands and Norway were collected on the day of delivery, while samples in Finland were mainly (74%) collected before the day of delivery, with an interval up to 31 days. Sample collection and processing may possibly have led to some in vitro complement activation. Importantly, such pre-analytical steps may impact on hemolytic activity of complement activity of samples or on complement activation fragments but does typically not impact on complement protein levels as measured by sandwich ELISA. If such effect may be present in a subset of the samples, then this is at least partially mitigated by the fact that samples for PE and controls were handled similarly. Furthermore, the focus on systemic levels in this investigation may not be fully representative of local effects in the placenta, leaving the question of cause and consequence in the etiology of PE open.

In conclusion, circulating levels of anti-FH and anti-C1q are not associated with the occurrence of PE. Circulating levels of C1q and FH are increased in healthy pregnancies as compared to nonpregnant controls. Importantly, circulating levels of FH are

decreased in PE as compared to control pregnancy. Exactly how C1q is involved in the processes of placentation and pregnancy as a whole is still under investigation, but a key aspect appears to be that C1q is mediating its effect in the absence of clear complement activation [9]. C1q deficiency is strongly associated with autoimmune disease SLE, as a result of insufficient cellular waste clearance [40]. The increased C1q levels found during pregnancy could be a way to avoid accumulation of waste from placental remodeling. Upregulation of FH during healthy pregnancy could be a way to counteract excess complement activation, but FH has also been shown to promote a tolerogenic phenotype in dendritic cells, hinting at a noncanonical function for FH [41]. Failure to increase FH level during pregnancy may therefore result in insufficient immune regulation contributing to development of PE. Further research will have to disclose what share of PE etiology can be ascribed to a disrupted equilibrium of complement activation and regulation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval for the three cohorts was obtained locally, from the Medical Research Ethics Committee of Leiden University Medical Center, the Coordinating Ethics Committee, Hospital District of Helsinki and Uusimaa or the Regional Committee for Medical Research Ethics Central Norway, respectively. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ME, M-LvdH, and LT designed the study. DD, NB, and CvdK performed the laboratory work. DD, AL, and LG analyzed the data. DD wrote the draft manuscript. All authors provided feedback to the manuscript and approved it.

Funding

LT has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 724517).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The Finnish Genetics of Pre-Eclampsia Consortium (FINNPEC) Core Investigator Group

The FINNPEC core investigator group consists of the following persons:

Hannele Laivuori (principal investigator)

- Medical and Clinical Genetics, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
- Institute for Molecular Medicine Finland, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland
- Department of Obstetrics and Gynecology, Tampere University Hospital and Tampere University, Faculty of Medicine and Health Technology, Tampere Center for Child, Adolescent, and Maternal Health Research, Tampere, Finland

Seppo Heinonen

- Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Eero Kajantie

- PEDEGO Research Unit, Medical Research Center Oulu, Oulu University Hospital and University of Oulu, Oulu, Finland
- Public Health Promotion Unit, National Institute for Health and Welfare, Helsinki and Oulu, Finland
- Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
- Department of Clinical and Molecular Medicine, Norwegian University of Health and Technology, Trondheim, Norway

Juha Kere

- Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden
- Folkhälsan Research Center and Stem Cells and Metabolism Research Program, University of Helsinki, Helsinki, Finland

Katja Kivinen

- Institute for Molecular Medicine Finland, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland

Anneli Pouta

- Department of Government Services, National Institute for Health and Welfare, Helsinki, Finland

References

1. S. Lisonkova, K. S. Joseph, Incidence of preeclampsia: Risk factors and outcomes associated with early- versus late-onset disease. *Am J Obstet Gynecol* **209**, 544.e541-544.e512 (2013).
2. C. W. Redman, I. L. Sargent, Latest advances in understanding preeclampsia. *Science* **308**, 1592-1594 (2005).
3. E. A. Steegers, P. von Dadelszen, J. J. Duvekot, R. Pijnenborg, Pre-eclampsia. *Lancet* **376**, 631-644 (2010).
4. M. J. Walport, Complement. First of two parts. *N Engl J Med* **344**, 1058-1066 (2001).
5. D. Ricklin, G. Hajishengallis, K. Yang, J. D. Lambris, Complement: A key system for immune surveillance and homeostasis. *Nature immunology* **11**, 785 (2010).
6. A. J. Nauta, L. A. Trouw, M. R. Daha, O. Tijsma, R. Nieuwland, W. J. Schwaeble, A. R. Gingras, A. Mantovani, E. C. Hack, A. Roos, Direct binding of c1q to apoptotic cells and cell blebs induces complement activation. *European journal of immunology* **32**, 1726-1736 (2002).
7. C. Agostinis, R. Bulla, C. Tripodo, A. Gismondi, H. Stabile, F. Bossi, C. Guarnotta, C. Garlanda, F. De Seta, P. Spessotto, A. Santoni, B. Ghebrehiwet, G. Girardi, F. Tedesco, An alternative role of c1q in cell migration and tissue remodeling: Contribution to trophoblast invasion and placental development. *J Immunol* **185**, 4420-4429 (2010).
8. J. Singh, A. Ahmed, G. Girardi, Role of complement component c1q in the onset of preeclampsia in mice. *Hypertension* **58**, 716-724 (2011).
9. C. Agostinis, F. Tedesco, R. Bulla, Alternative functions of the complement protein c1q at embryo implantation site. *J Reprod Immunol* **119**, 74-80 (2017).
10. R. A. van Schaarenburg, C. Magro-Checa, J. A. Bakker, Y. K. Teng, I. M. Bajema, T. W. Huizinga, G. M. Steup-Beekman, L. A. Trouw, C1q deficiency and neuropsychiatric systemic lupus erythematosus. *Front Immunol* **7**, 647 (2016).
11. L. A. Trouw, A. A. Bengtsson, K. A. Gelderman, B. Dahlbäck, G. Sturfelt, A. M. Blom, C4b-binding protein and factor h compensate for the loss of membrane-bound complement inhibitors to protect apoptotic cells against excessive complement attack. *Journal of Biological Chemistry* **282**, 28540-28548 (2007).
12. A. I. Lokki, J. Heikkinen-Eloranta, H. Jarva, T. Saisto, M. L. Lokki, H. Laivuori, S. Meri, Complement activation and regulation in preeclamptic placenta. *Front Immunol* **5**, 312 (2014).
13. A. I. Lokki, J. Heikkinen-Eloranta, Pregnancy induced tma in severe preeclampsia results from complement-mediated thromboinflammation. *Hum Immunol* **82**, 371-378 (2021).
14. M. A. Dragon-Durey, C. Blanc, M. C. Marinozzi, R. A. van Schaarenburg, L. A. Trouw, Autoantibodies against complement components and functional consequences. *Mol Immunol* **56**, 213-221 (2013).
15. M. A. Seelen, L. A. Trouw, M. R. Daha, Diagnostic and prognostic significance of anti-c1q antibodies in systemic lupus erythematosus. *Curr Opin Nephrol Hypertens* **12**, 619-624 (2003).
16. L. A. Trouw, T. W. Groeneveld, M. A. Seelen, J. M. Duijs, I. M. Bajema, F. A. Prins, U. Kishore, D. J. Salant, J. S. Verbeek, C. van Kooten, M. R. Daha, Anti-c1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular c1q-containing immune complexes. *J Clin Invest* **114**, 679-688 (2004).

17. F. S. van de Bovenkamp, D. J. Dijkstra, C. van Kooten, K. A. Gelderman, L. A. Trouw, Circulating c1q levels in health and disease, more than just a biomarker. *Mol Immunol* **140**, 206-216 (2021).
18. A. Foltyn Zadura, A. A. Memon, L. Stojanovich, C. Perricone, F. Conti, G. Valesini, G. Bogdanovic, A. Hillarp, Y. Shoenfeld, J. Sundquist, J. Leffler, P. J. Svensson, L. A. Trouw, A. M. Blom, Factor h autoantibodies in patients with antiphospholipid syndrome and thrombosis. *J Rheumatol* **42**, 1786-1793 (2015).
19. C. Blanc, S. K. Togarsimalemath, S. Chauvet, M. Le Quintrec, B. Moulin, M. Buchler, T. S. Jokiranta, L. T. Roumenina, V. Fremeaux-Bacchi, M. A. Dragon-Durey, Anti-factor h autoantibodies in c3 glomerulopathies and in atypical hemolytic uremic syndrome: One target, two diseases. *J Immunol* **194**, 5129-5138 (2015).
20. H. Vitkova, J. Jiskra, D. Springer, Z. Limanova, Z. Telicka, J. Bartakova, M. Trendelenburg, E. Potlukova, Anti-c1q autoantibodies are linked to autoimmune thyroid disorders in pregnant women. *Clin Exp Immunol* **186**, 10-17 (2016).
21. I. V. Menzhinskaya, L. V. Van'ko, M. M. Kashentseva, P. A. Kiryushchenkov, G. T. Sukhikh, Incidence of autoantibodies to c1q complement component in women with miscarriages and autoantibodies to phospholipids and chorionic gonadotropin. *Bull Exp Biol Med* **160**, 260-263 (2015).
22. A. Daponte, E. Deligeoroglou, S. Pournaras, C. Hadjichristodoulou, A. Garas, F. Anastasiadou, I. E. Messinis, Interleukin-15 (il-15) and anti-c1q antibodies as serum biomarkers for ectopic pregnancy and missed abortion. *Clin Dev Immunol* **2013**, 637513 (2013).
23. G. Moroni, A. Doria, E. Giglio, E. Imbasciati, C. Tani, M. Zen, F. Strigini, B. Zaina, A. Tincani, M. Gatto, F. de Liso, C. Grossi, P. L. Meroni, G. Cabiddu, P. Messa, P. Ravani, M. Mosca, Maternal outcome in pregnant women with lupus nephritis. A prospective multicenter study. *J Autoimmun* **74**, 194-200 (2016).
24. T. Jääskeläinen, S. Heinonen, E. Kajantie, J. Kere, K. Kivinen, A. Pouta, H. Laivuori, Cohort profile: The finnish genetics of pre-eclampsia consortium (finnpec). *BMJ Open* **6**, e013148 (2016).
25. R. Lubbers, J. S. Sutherland, D. Goletti, R. A. de Paus, C. H. M. van Moorsel, M. Veltkamp, S. M. T. Vestjens, W. J. W. Bos, L. Petrone, F. Del Nonno, . . . L. A. Trouw, Complement component c1q as serum biomarker to detect active tuberculosis. *Front Immunol* **9**, 2427 (2018).
26. S. M. Yie, R. N. Taylor, C. Librach, Low plasma hla-g protein concentrations in early gestation indicate the development of preeclampsia later in pregnancy. *Am J Obstet Gynecol* **193**, 204-208 (2005).
27. R. Hackmon, A. Koifman, H. Hyodo, H. Glickman, E. Sheiner, D. E. Geraghty, Reduced third-trimester levels of soluble human leukocyte antigen g protein in severe preeclampsia. *Am J Obstet Gynecol* **197**, 255.e251-255 (2007).
28. L. Marozio, A. Garofalo, P. Berchialla, A. M. Tavella, L. Salton, F. Cavallo, C. Benedetto, Low expression of soluble human leukocyte antigen g in early gestation and subsequent placenta-mediated complications of pregnancy. *J Obstet Gynaecol Res* **43**, 1391-1396 (2017).
29. M. Ghaebi, M. Nouri, A. Ghasemzadeh, L. Farzadi, F. Jadidi-Niaragh, M. Ahmadi, M. Yousefi, Immune regulatory network in successful pregnancy and reproductive failures. *Biomed Pharmacother* **88**, 61-73 (2017).

30. S. Wedenoja, M. Yoshihara, H. Teder, H. Sariola, M. Gissler, S. Katayama, J. Wedenoja, I. M. Häkkinen, S. Ezer, N. Linder, . . . J. Kere, Fetal hla-g mediated immune tolerance and interferon response in preeclampsia. *EBioMedicine* **59**, 102872 (2020).

31. M. E. Benoit, E. V. Clarke, P. Morgado, D. A. Fraser, A. J. Tenner, Complement protein c1q directs macrophage polarization and limits inflammasome activity during the uptake of apoptotic cells. *J Immunol* **188**, 5682-5693 (2012).

32. K. Jia, L. Ma, S. Wu, W. Yang, Serum levels of complement factors c1q, bb, and h in normal pregnancy and severe pre-eclampsia. *Med Sci Monit* **25**, 7087-7093 (2019).

33. Y. D. He, B. N. Xu, M. L. Wang, Y. Q. Wang, F. Yu, Q. Chen, M. H. Zhao, Dysregulation of complement system during pregnancy in patients with preeclampsia: A prospective study. *Mol Immunol* **122**, 69-79 (2020).

34. Z. Derzsy, Z. Prohászka, J. Rigó, Jr., G. Füst, A. Molvarec, Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol* **47**, 1500-1506 (2010).

35. M. C. Hoffman, K. K. Rumer, A. Kramer, A. M. Lynch, V. D. Winn, Maternal and fetal alternative complement pathway activation in early severe preeclampsia. *Am J Reprod Immunol* **71**, 55-60 (2014).

36. A. M. Lynch, J. R. Murphy, T. Byers, R. S. Gibbs, M. C. Neville, P. C. Giclas, J. E. Salmon, V. M. Holers, Alternative complement pathway activation fragment bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol* **198**, 385.e381-389 (2008).

37. J. E. Salmon, C. Heuser, M. Triebwasser, M. K. Liszewski, D. Kavanagh, L. Roumenina, D. W. Branch, T. Goodship, V. Fremeaux-Bacchi, J. P. Atkinson, Mutations in complement regulatory proteins predispose to preeclampsia: A genetic analysis of the promisse cohort. *PLoS Med* **8**, e1001013 (2011).

38. Y. Xin, E. Hertle, C. J. H. van der Kallen, C. G. Schalkwijk, C. D. A. Stehouwer, M. M. J. van Greevenbroek, Longitudinal associations of the alternative and terminal pathways of complement activation with adiposity: The codam study. *Obes Res Clin Pract* **12**, 286-292 (2018).

39. J. M. Moreno-Navarrete, R. Martínez-Barricarte, V. Catalán, M. Sabater, J. Gómez-Ambrosi, F. J. Ortega, W. Ricart, M. Blüher, G. Frühbeck, S. Rodríguez de Cordoba, J. M. Fernández-Real, Complement factor h is expressed in adipose tissue in association with insulin resistance. *Diabetes* **59**, 200-209 (2010).

40. G. C. Tsokos, M. S. Lo, P. Costa Reis, K. E. Sullivan, New insights into the immunopathogenesis of systemic lupus erythematosus. *Nat Rev Rheumatol* **12**, 716-730 (2016).

41. R. Olivar, A. Luque, S. Cárdenas-Brito, M. Naranjo-Gómez, A. M. Blom, F. E. Borràs, S. Rodriguez de Córdoba, P. F. Zipfel, J. M. Aran, The complement inhibitor factor h generates an anti-inflammatory and tolerogenic state in monocyte-derived dendritic cells. *J Immunol* **196**, 4274-4290 (2016).

CHAPTER 5

Anti-C1q autoantibodies may not serve as an adequate biomarker for lung manifestations in systemic sclerosis: a single-center, cross-sectional study

Douwe J. Dijkstra[1], Sophie I. E. Liem[2], Nina M. van Leeuwen[2], Cynthia M. Fehres[2], Jeska K. de Vries-Bouwstra[2], Leendert A. Trouw[1]

1. Department of Immunology, Leiden University Medical Center, Leiden, the Netherlands
2. Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands

Published as a letter to the editor in: British Journal of Dermatology (2021), doi: *10.1111/bjd.20412*

Introduction

Systemic sclerosis (SSc) is a rheumatic disease characterized by fibrosis in the skin and internal organs, including the lungs, kidneys and gastrointestinal tract, as well as microvascular injuries [1]. Deaths related to SSc are mostly attributed to involvement of the lungs and the heart, with pulmonary fibrosis and pulmonary arterial hypertension (PAH) being the most common lung conditions associated with SSc [2]. Although the disease mechanism is not fully understood, it is clear that the pathogenesis of SSc involves a considerable immune component as evidenced by the presence of antinuclear autoantibodies (ANA) in the vast majority of patients [3]. ANA prominently target centromere proteins, topoisomerase and RNA polymerase III, and are often used as predictors of disease outcome and organ complications [4]. Recently, autoantibodies targeting complement component C1q were suggested to be predictive of pulmonary fibrosis or PAH [5]. As it would be highly desirable to have a biomarker for the most severe clinical presentation of SSc, we set out to replicate these findings in a Dutch cohort.

Materials & methods

In this study, sera of 188 patients with SSc and 80 healthy controls were tested for the presence of anti-C1q autoantibodies. Patients were mostly female (149 of 188, 79%) and the median age was 56.6 years (interquartile range 46.8–65.5). Diffuse cutaneous SSc was present in 39 patients (21%). All patients fulfilled the American College of Rheumatology/ European League Against Rheumatism 2013 SSc criteria, had a clinical diagnosis of SSc, and were included in the Combined Care in SSc cohort at Leiden University Medical Center [6]. Serum samples originated between 2012 and 2018. Presence of anti-C1q autoantibodies was determined in all sera by QUANTA Lite Anti-C1q ELISA (Inova Diagnostics, San Diego, CA, USA), using the cutoff for positivity of 20 units as recommended by the manufacturer.

Results & conclusion

In total, 21 (11%) patients with SSc and 10 (13%) healthy controls were assessed as positive for anti-C1q autoantibodies (Figure 1A). The prevalence of anti-C1q autoantibodies in healthy controls is not unexpected, as previous studies have reported frequencies between 2% and 13.5% [7, 8]. We compared the occurrence of several clinical parameters, including interstitial lung disease (ILD) assessed with high-resolution computed tomography, ILD combined with a forced vital capacity (FVC) below 80% of predicted, and PAH, between anti-C1q-positive and anti-C1q-negative patients with SSc. PAH was defined as a mean pulmonary arterial pressure $\geq$ 25 mmHg at rest as assessed

by right heart catheterization (RHC), including presence of precapillary pulmonary hypertension, defined by a pulmonary capillary wedge pressure ≤ 15 mmHg, and a pulmonary vascular resistance > 3 Wood units on RHC. All patients with suspicion for PAH were referred for RHC. No significant differences were observed in the incidence of these SSc-related lung conditions between the patients who were anti-C1q positive or negative (Figure 1B).

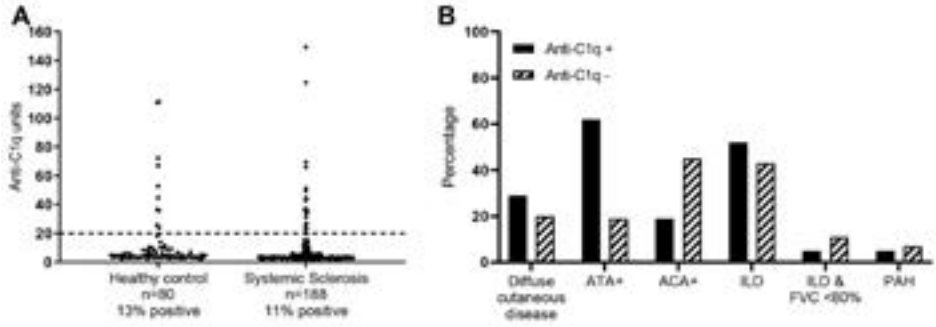


Figure 1. Anti-C1q in systemic sclerosis. (A) Anti-C1q autoantibodies in healthy controls and patients with systemic sclerosis, with the cutoff for positivity (20 units) indicated by the dotted line. (B) Percentage of diffuse cutaneous disease, presence of anti-topoisomerase antibodies (ATA) and anti-centromere antibodies (ACA), interstitial lung disease (ILD), clinically relevant ILD and pulmonary arterial hypertension (PAH) within anti-C1q-positive and anti-C1q-negative patients. FVC, forced vital capacity.

Diffuse cutaneous disease was present in 33 of 167 (20%) anti-C1q-negative patients and in six of 21 (29%) anti-C1q-positive patients, a nonsignificant difference. Presence of anti-topoisomerase antibodies (ATA) and anti-centromere antibodies (ACA) was determined as part of diagnostics, with ATA often correlating with more severe disease. Interestingly, ATA were present at a higher rate in anti-C1q-positive patients (13 of 21, 62% vs 32 of 167, 19% in anti-C1q negative patients; $p < 0.001$), while there was no significant difference for ACA. Moreover, anti-C1q autoantibodies were found at a higher frequency in male than in female patients (nine of 39, 23% vs 12 of 149, 8%; $p = 0.008$).

The original study into anti-C1q autoantibodies in SSc found significantly more pulmonary fibrosis (55% vs 28.8%) and more diffuse cutaneous SSc in anti-C1q-positive than anti-C1q-negative patients [5]. These findings suggested more severe disease in anti-C1q-positive patients. While in the present study ILD was found to be somewhat enriched in anti-C1q-positive patients (11 of 21, 52% in anti-C1q-positive patients vs 71 of 167, 43% in anti-C1q-negative patients), this finding held no statistical significance. When investigating clinically relevant ILD (combined with FVC < 80%), the

prevalence was even lower in anti-C1q-positive patients, and the same holds true for PAH. Furthermore, the observed association of anti-C1q with ATA, which is already reported to associate with lung complications, would detract from any added value of anti-C1q in SSc diagnostics. We therefore conclude that the presence of anti-C1q autoantibodies in our Dutch cohort is not correlated with SSc-related lung conditions. The aforementioned differences could be related to nonidentical patient populations in the respective studies. Compared with Liaskos *et al.*, the current study includes a higher number of patients with SSc and a higher prevalence of ILD, but lower percentages of patients with diffuse cutaneous SSc and PAH [5]. Nonetheless, the present study does not support a prognostic value for anti-C1q autoantibodies in SSc or its related lung conditions.

Author Contributions

D.J. Dijkstra: Conceptualization (equal); Investigation (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal). S.I.E. Liem: Formal analysis (equal); Writing-review & editing (equal). N.M. van Leeuwen: Formal analysis (equal); Writing-review & editing (equal). C.M. Fehres: Conceptualization (equal); Writing-review & editing (equal). J.K. de Vries-Bouwstra: Writing-review & editing (equal). L.A. Trouw: Conceptualization (equal); Writing-review & editing (equal).

References

1. C. P. Denton, D. Khanna, Systemic sclerosis. *The Lancet* **390**, 1685-1699 (2017).
2. A. J. Tyndall, B. Bannert, M. Vonk, P. Airò, F. Cozzi, P. E. Carreira, D. F. Bancel, Y. Allanore, U. Müller-Ladner, O. Distler, Causes and risk factors for death in systemic sclerosis: A study from the eular scleroderma trials and research (eustar) database. *Annals of the rheumatic diseases* **69**, 1809-1815 (2010).
3. G. A. Salazar, S. Assassi, F. Wigley, L. Hummers, J. Varga, M. Hinchcliff, D. Khanna, E. Schiopu, K. Phillips, D. E. Furst (2015) Antinuclear antibody-negative systemic sclerosis. in *Seminars in arthritis and rheumatism* (Elsevier), pp 680-686.
4. S. I. Nihtyanova, C. P. Denton, Autoantibodies as predictive tools in systemic sclerosis. *Nature Reviews Rheumatology* **6**, 112 (2010).
5. C. Liaskos, S. Rentouli, T. Simopoulou, A. Gkoutzourelas, G. Norman, A. Brotis, I. Alexiou, C. Katsiari, D. Bogdanos, L. Sakkas, Anti-c1q autoantibodies are frequently detected in patients with systemic sclerosis associated with pulmonary fibrosis. *British Journal of Dermatology* **181**, 138-146 (2019).
6. J. Meijs, A. A. Schouffoer, N. A. Marsan, L. J. Kroft, T. Stijnen, M. K. Ninaber, T. W. Huizinga, T. P. V. Vlieland, J. K. de Vries-Bouwstra, Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. *RMD open* **2**, e000159 (2016).
7. F. J. Beurskens, R. A. van Schaarenburg, L. A. Trouw, C1q, antibodies and anti-c1q autoantibodies. *Molecular immunology* **68**, 6-13 (2015).
8. C. Magro-Checa, R. Schaarenburg, H. Beaart, T. Huizinga, G. Steup-Beekman, L. Trouw, Complement levels and anti-c1q autoantibodies in patients with neuropsychiatric systemic lupus erythematosus. *Lupus* **25**, 878-888 (2016).

CHAPTER 6

Human anti-C1q autoantibodies bind specifically to solid-phase C1q and enhance phagocytosis but not complement activation

Douwe J. Dijkstra[1], Fleur S. van de Bovenkamp[1,2], Leoni Abendstein[3], Rob Zuijderduijn[1], Jos Pool[1], Cynthia S.M. Kramer[1], Linda M. Slot[4], Jan W. Drijfhout[1], Lisanne de Vor[5], Kyra A. Gelderman[6], Suzan H.M. Rooijakkers[5], Arnaud Zaldumbide[3], Gestur Vidarsson[7], Thomas H. Sharp[3], Paul W.H.I. Parren[1,8], Leendert A. Trouw[1]

1. Department of Immunology, Leiden University Medical Center, Leiden, the Netherlands
2. Lava Therapeutics, Utrecht, the Netherlands
3. Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, the Netherlands
4. Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
5. Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands
6. Sanquin Diagnostic Services, Amsterdam, the Netherlands
7. Department of Experimental Immunohematology, Sanquin Research, Amsterdam, the Netherlands
8. Gyes BV, Naarden, the Netherlands

Proceedings of the National Academy of Sciences of the United States of America (2023),
doi: *10.1073/pnas.2310666120*

Abstract

Autoantibodies directed against complement component C1q are commonly associated with autoimmune diseases, especially systemic lupus erythematosus. Importantly, these anti-C1q autoantibodies are specific for ligand-bound, solid-phase C1q and do not bind to fluid-phase C1q. In patients with anti-C1q, C1q levels are in the normal range, and the autoantibodies are thus not depleting. To study these human anti-C1q autoantibodies at the molecular level, we isolated C1q-reactive B cells and recombinantly produced nine monoclonal antibodies (mAbs) from four different healthy individuals. The isolated mAbs were of the IgG isotype, contained extensively mutated variable domains, and showed high affinity to the collagen-like region of C1q. The anti-C1q mAbs exclusively bound solid-phase C1q in complex with its natural ligands, including immobilized or antigen-bound IgG, IgM or CRP, and necrotic cells. Competition experiments reveal that at least 2 epitopes, also targeted by anti-C1q antibodies in sera from SLE patients, are recognized. Electron microscopy with hexameric IgG-C1q immune complexes demonstrated that multiple mAbs can interact with a single C1q molecule and identified the region of C1q targeted by these mAbs. The opsonization of immune complexes with anti-C1q greatly enhanced Fc-receptor-mediated phagocytosis but did not increase complement activation. We conclude that human anti-C1q autoantibodies specifically bind neo-epitopes on solid-phase C1q, which results in an increase in Fc-receptor-mediated effector functions that may potentially contribute to autoimmune disease immunopathology.

Significance statement

Autoantibodies against complement component C1q are associated with several autoimmune diseases. These autoantibodies specifically bind to ligand-bound, solid-phase C1q, but not fluid-phase C1q. We identified nine human anti-C1q clones from healthy individuals and produced them recombinantly. We found that these autoantibodies recognize the collagen-like region of solid-phase C1q, when C1q is in complex with a range of its natural ligands. Electron microscopy revealed that multiple antibodies bind a single C1q molecule and further identified the region of C1q targeted by these mAbs. Binding of anti-C1q does not increase complement activation on immune complexes; however, we demonstrated that anti-C1q autoantibodies bound to solid-phase C1q can activate immune cells by engaging Fc-receptors. Anti-C1q autoantibodies may thereby contribute to pathological processes in autoimmune disease.

Introduction

The complement system plays an important role in both innate immune defense and the development of adaptive immune responses. In addition, complement is involved in the clearance of immune complexes, cellular debris, and tissue remodeling [1]. In the classical pathway of complement activation, the recognition molecule C1q binds a wide array of ligands, including target-bound immunoglobulins (Ig) and C-reactive protein (CRP), which trigger the activation of the C1 enzymes C1r and C1s and propagation of the complement cascade. The function of C1q in normal physiology is underscored by the clinical presentation of C1q-deficient patients with infections and systemic lupus erythematosus (SLE)-like disease [2]. Excessive complement activation is thought to be a major pathological mechanism of several rheumatic autoimmune diseases including SLE [3].

Autoantibodies targeting specific complement proteins have been detected in several autoimmune disorders [4]. Anti-C1q autoantibodies have been found to be prevalent in hypocomplementemic urticarial vasculitis syndrome (HUVS; 100% of the patients) and in SLE (around 40% of the patients)(reviewed in refs. [5, 6]). In SLE, the presence of anti-C1q autoantibodies is associated with lupus nephritis [7-9]. Interestingly, anti-C1q autoantibodies are also present in a substantial fraction of the healthy population, indicating that anti-C1q autoantibodies may only be pathogenic in certain circumstances [10, 11]. Indeed, experiments in mice indicate that anti-C1q autoantibodies only induce damage to the kidney when C1q is already present on deposited immune complexes [7]. Anti-C1q autoantibodies are measured in routine diagnostics, but require tailored experimental conditions to avoid binding of immune complexes to the immobilized C1q [12]. The detection of anti-C1q autoantibodies is therefore performed in buffers containing a high concentration of NaCl, as this prevents ligand binding of C1q, while largely retaining the binding of anti-C1q autoantibodies [13].

The molecular characteristics of anti-C1q autoantibodies are currently unknown, but studies on patient sera indicated a specificity for solid-phase C1q [14]. The term solid-phase C1q is used in the literature to describe C1q bound to any ligand or surface [14]. During complement initiation, C1q undergoes a conformational change upon binding to its ligands, which reveals neo-epitopes to which antibodies specific for solid-phase C1q can bind [15] (Figure 1A). Several studies using serum from SLE patients have reported that anti-C1q autoantibodies mainly target the collagen-like region (CLR) as opposed to the globular head (GH) domains [16, 17]. A few reports described that autoantibodies against GH polypeptides can also be present in some individuals [18, 19], and another report described epitope mapping using linear peptides [20].

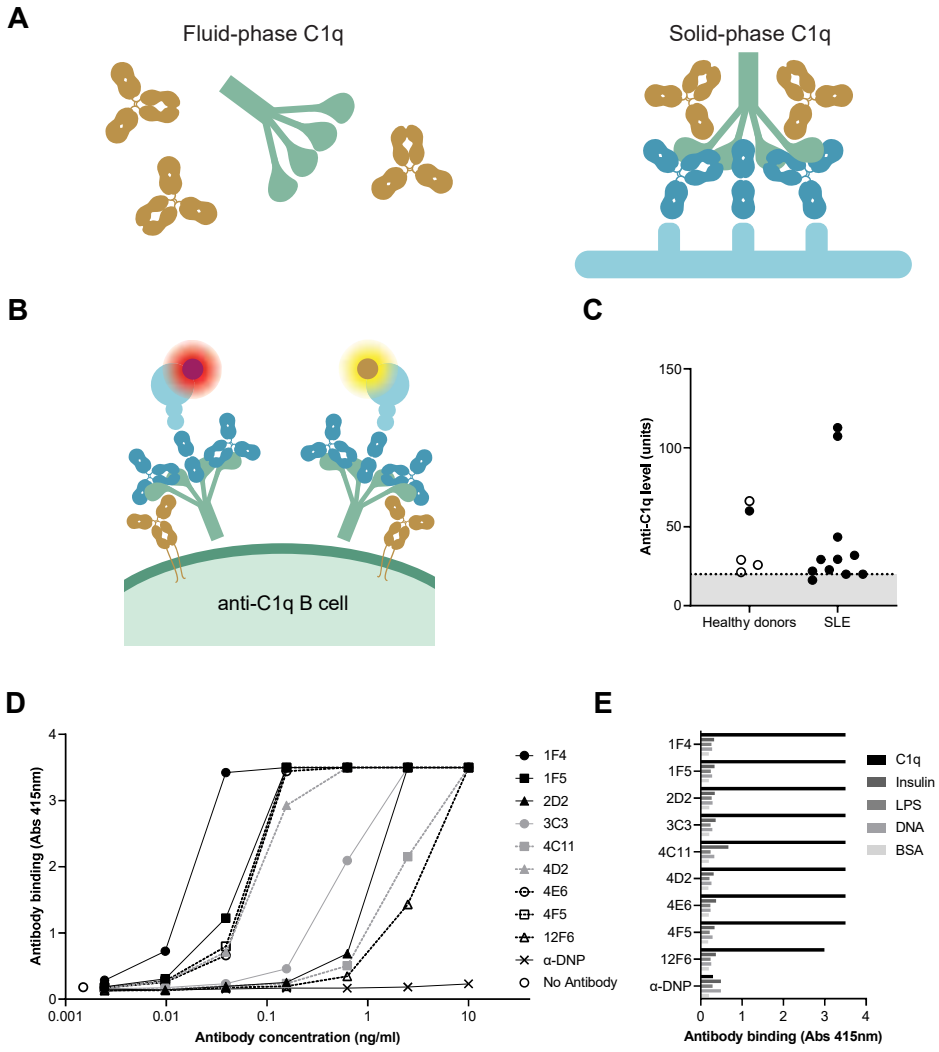


Figure 1. Human solid-phase C1q autoantibodies were cloned from several healthy donors. (A) Anti-C1q antibodies do not bind to fluid-phase C1q (Left) but do bind to solid-phase C1q (Right), which is the conformation C1q adopts upon binding to a ligand or surface. (B) Schematic overview of the staining complex used for sorting anti-C1q-specific B cells. C1q (green) is in a solid-phase conformation in complex with hexameric anti-DNP IgG (dark blue), which binds fluorescently labeled streptavidin via a DNP- and biotin-containing peptide (light blue). Streptavidin was labeled with either PE (yellow) or AlexaFluor647 (red). (C) Anti-C1q levels of selected healthy donors and SLE patient sera (open circles represent donors from whom anti-C1q mAbs were successfully isolated). The dotted line marks cut-off for positivity according to the manufacturer of the kit (QUANTA Lite Anti-C1q ELISA; Werfen). (D) Dose–response for recombinant mAb–biotin for binding to C1q assessed in homemade C1q-coated ELISA, detected with streptavidin-HRP. (E) Binding of 2 µg/mL anti-C1q mAb to well-known ligands of polyreactive mAbs, chimeric anti-DNP IgG1 (clone G2a2) was used as control mAb. For panels (D) and E, a representative of at least two independent experiments was shown.

Most studies characterizing anti-C1q autoantibodies have focused on polyclonal antibodies from serum of human donors, either patients or healthy individuals. An investigation into monoclonal human anti-C1q to analyze anti-C1q autoantibody responses on a molecular and functional level is therefore warranted. We set out to isolate single anti-C1q-positive B cells, clone them, and recombinantly produce human monoclonal antibodies (mAbs). In total, we generated nine anti-C1q human mAbs, recognizing two distinct epitopes on the CLR of solid-phase C1q. We demonstrate that the epitopes targeted by the anti-C1q mAbs constitute the same epitopes targeted by anti-C1q autoantibodies present in SLE sera. Our study may lead to diagnostics or therapeutics for the treatment of complement-mediated inflammatory and autoimmune diseases and may provide insight into the immunopathological processes that drive lupus nephritis.

Results

Isolation of anti-C1q-positive B cells from peripheral blood

To select possible donors for C1q-reactive B cells, sera from healthy donors and SLE patients were screened for the presence of anti-C1q autoantibodies. We identified five healthy donors and 11 SLE patients with anti-C1q autoantibodies, who were used for isolating B cells (Figure 1C). Single B cells from these anti-C1q positive donors were FACS sorted for C1q reactivity using soluble immunocomplexes presenting C1q in solid-phase. Therefore, the cells were stained using C1q bound to pre-formed IgG hexamers against DNP which were detected with DNP-biotin in complex with either PE or AlexaFluor647. We sorted the cells double positive for PE and AlexaFluor647. Sorted cells were cultured and supernatant was screened for anti-C1q IgG, resulting in the successful identification of nine unique anti-C1q mAbs from healthy donors.

Variable domains of anti-C1q mAbs were sequenced and analyzed in IMGT V-quest to determine mutational load and V(D)J gene usage. The number of mutations in the V genes (up to 37) suggests that several of the isolated anti-C1q mAbs had undergone substantial somatic hypermutation (Table 1). The antibody variable domain analysis showed diversity in putative V(D)J gene usage, even between clones isolated from the same donor. Only mAbs 4D2 and 4E6 from donor 2 were highly similar and likely derived from a common ancestor B cell.

Table 1. Genetic characteristics of human anti-C1q mAbs as analyzed by IMGT V-quest [21]. CDR3, Complementarity-Determining Region 3; AA, amino acid.

Heavy Chain							
Clone name	**Donor**	**V-gene**	**V-gene mutations**	**V-gene identity (%)**	**D-gene**	**J-gene**	**CDR3 AA sequence**
2D2	1	IGHV3-74*03 F	14	95.14	IGHD6-13*01 F	IGHJ6*02 F	ARGPHISSWFSDYSYAMDV
1F4	2	IGHV3-30*01 F	37	87.2	IGHD3-16*01 F	IGHJ4*02 F	ARGDCGDVTCSLDS
4C11	2	IGHV1-8*01 F	3	99.0	IGHD3-3*01 F	IGHJ6*02 F	AKISAIFGVVQSGYYYYGMDV
4D2	2	IGHV1-18*01 F	37	87.2	IGHD3-22*01 F	IGHJ3*01 F	ARVNNANFYDRNGYFEGRTRTEAFDF
4E6	2	IGHV1-18*01 F	35	87.9	IGHD3-22*01 F	IGHJ3*01 F	ARVNNADYYDSSGYFQGRTRTEAFDF
4F5	2	IGHV4-34*01 F	10	96.5	IGHD3-22*01 F	IGHJ6*02 F	ARERGGHYYEDIGYYGDPGMDV
1F5	3	IGHV1-18*01 F	34	88.2	IGHD6-6*01 F	IGHJ4*02 F	SINSQLAY
3C3	4	IGHV4-39*01 F	16	94.5	IGHD4-17*01 F	IGHJ4*02 F	ASQRDHGDYVRGPDY
12F6	4	IGHV3-21*02 F	1	99.7	IGHD1-26*01 F	IGHJ3*02 F	ARISLVEWELAGYDAFDI
Light Chain							
Clone name	**Donor**	**V-gene**	**V-gene mutations**	**V-gene identity (%)**	**kappa / lambda**	**J-gene**	**CDR3 AA sequence**
2D2	1	IGKV2-28*01 F	3	99.0	kappa	IGKJ1*01 F	MQALQTPPA
1F4	2	IGLV2-11*01 F	23	92.0	lambda	IGLJ1*01 F	CSYGDRNPFV
4C11	2	IGKV2-28*01 F	4	98.6	kappa	IGKJ1*01 F	MQALQTPKT
4D2	2	IGLV2-14*01 F	14	95.1	lambda	IGLJ1*01 F	SSYSSLSPCV
4E6	2	IGLV2-14*01 F	18	93.8	lambda	IGLJ1*01 F	SSYTSLTPCV
4F5	2	IGKV3-20*01 F	8	97.2	kappa	IGKJ4*01 F	QQYGSSPRN
1F5	3	IGKV2-30*01 F	15	94.9	kappa	IGKJ1*01 F	MQGTHWPRT
3C3	4	IGLV1-40*01 F	13	95.5	lambda	IGLJ2*01 F	QSYDSNLSVV
12F6	4	IGLV1-47*01 F	0	100	lambda	IGLJ2*01 F	AAWDDSLSGVV

Anti-C1q mAbs were expressed recombinantly in the eukaryotic Expi293 expression system and purified for further analysis. Binding of the mAbs in C1q-coated enzyme-linked immunosorbent assay (ELISA) varied greatly, with more than 100-fold difference in mAb binding to C1q comparing the strongest and the weakest binder (Figure 1D). To exclude the possibility that the selected mAbs were polyreactive, binding to several well-known targets of (polyreactive) autoantibodies was tested in ELISA. For all anti-C1q

mAbs, no binding to single-stranded DNA, lipopolysaccharide (LPS), insulin, or BSA was observed, indicating specificity of these mAbs to C1q (Figure 1E). The avidity of isolated mAbs to C1q was quantified by surface plasmon resonance (SPR). Using solid-phase C1q on the chip and flowing the antibodies in fluid-phase, we observed a strong avidity with KD values of 6 to 23 nM (Table 2).

Table 2. Avidity of mAbs for C1q as determined by surface plasmon resonance.

mAb	K_D (M)	k_a (1/Ms)	k_d (1/s)
1F4	8.1×10^{-9}	1.4×10^{5}	1.1×10^{-3}
1F5	2.2×10^{-8}	1.1×10^{5}	2.4×10^{-3}
2D2	2.0×10^{-8}	9.7×10^{4}	2.0×10^{-3}
3C3	2.0×10^{-8}	6.3×10^{4}	1.2×10^{-3}
4C11	2.3×10^{-8}	1.8×10^{5}	4.3×10^{-3}
4D2	2.3×10^{-8}	9.9×10^{4}	2.3×10^{-3}
4E6	2.1×10^{-8}	1.4×10^{5}	2.9×10^{-3}
4F5	6.0×10^{-9}	1.4×10^{5}	8.4×10^{-4}
12F6	2.3×10^{-8}	6.6×10^{4}	1.5×10^{-3}

Human monoclonal anti-C1q autoantibodies bind selectively to solid-phase C1q

As anti-C1q autoantibodies detectable in the serum of healthy individuals and autoimmune patients are hypothesized to be specific for solid-phase C1q, binding of mAbs to coated, solid-phase C1q in the absence or presence of fluid-phase C1q was analyzed by ELISA. Use of 20 µg/mL purified C1q as a competitor did not yield any notable inhibition of mAbs binding to solid-phase C1q (Figure 2A). Adding normal, C1q-containing, serum also did not result in inhibition for the majority of mAbs. Only four of the mAbs, most notably mAb 4F5, were partially inhibited by C1q in serum at high (25%) concentration (Figure 2B and 2C). Neither the full C1qr2s2 complex (denoted C1 hereafter), nor C1q-depleted serum inhibited binding of the anti-C1q mAbs (Figure 2D). Mouse mAb 4A4B11, which is not specific for solid-phase C1q, was strongly inhibited by fluid-phase C1q as expected.

Finally, we studied the preferential binding of anti-C1q mAbs to solid-phase C1q in detail, without potential interference of bivalent binding or Fc-mediated C1q interactions. For this purpose, we engineered antibodies for mAbs 1F4, 1F5, and 4F5 with an Fc domain with mutations that abrogate C1q binding and that contain only a single C1q-binding Fab arm. In ELISA, binding of these anti-C1q mAbs to coated solid-phase C1q was analyzed in the presence of equal amounts of either fluid-phase C1q or C1q on beads (solid-phase). Binding for all three mAbs was significantly inhibited only by C1q on beads, but importantly not by fluid-phase C1q or by human albumin on beads or in fluid-phase (Figure 2E). Altogether, these anti-C1q mAbs show a strong selectivity toward binding to solid-phase C1q.

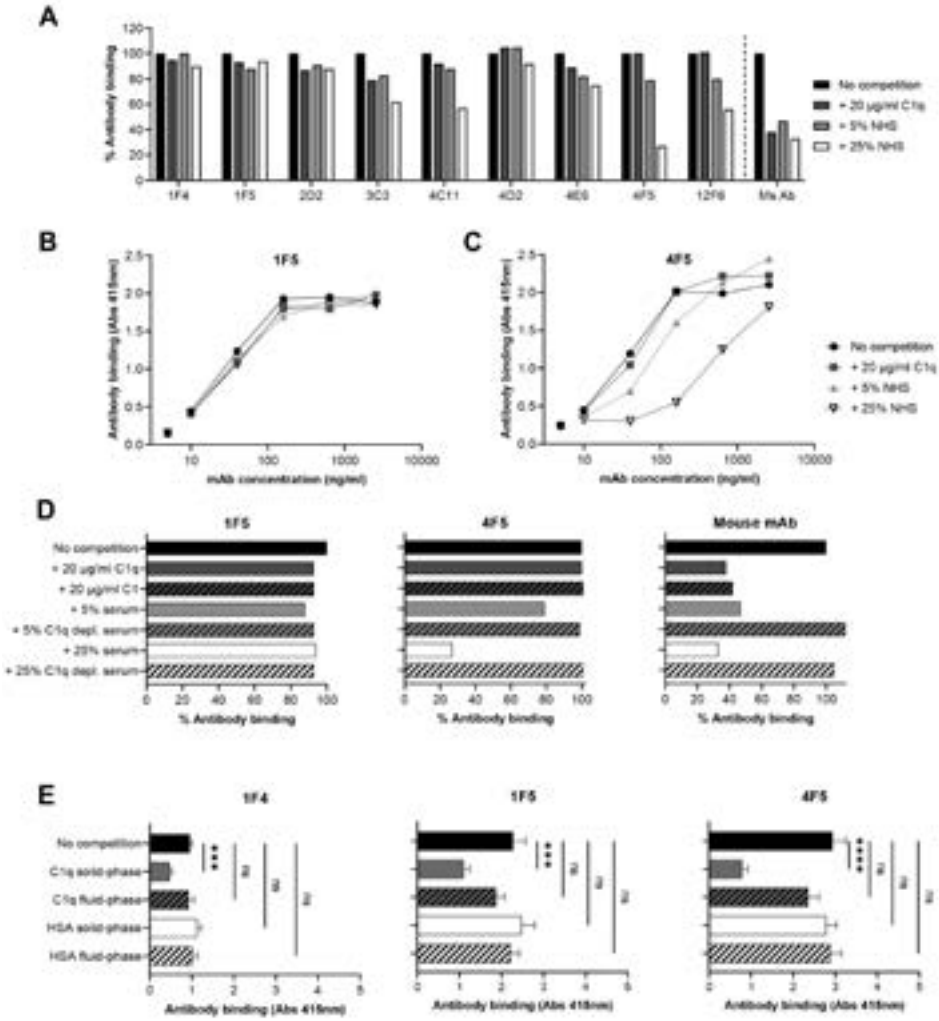


Figure 2. Binding of anti-C1q mAbs in ELISA in the presence of fluid-phase and solid-phase C1q. (A) Fluid-phase C1q competition measured in ELISA for all mAbs at a concentration at the top of the linear range for the specific mAb, to ensure competition could be detected optimally. Mouse mAb 4A4B11, which binds C1q but is not specific for solid-phase C1q, was included as a control. The measurements were performed at mAb concentrations of 160 ng/mL (1F5, 4F5, mouse mAb 4A4B11), 640 ng/mL (3C3, 4D2, 4E6), 2,560 ng/mL (1F4, 2D2, 4C11), or 10,240 ng/mL (12F6). Data shown are representative for two independent experiments. (B and C) Example titrations of competition assay with fluid phase purified C1q or NHS, for mAbs 1F5 (which shows no competition from high-concentration fluid-phase C1q) and 4F5 (which shows some competition from 25% NHS). (D) Competition with fluid-phase purified C1 or C1q, NHS, or C1q-depleted serum for selected anti-C1q mAbs 1F5, 4F5 and mouse mAb 4A4B11. (E) Anti-C1q mAb binding (monovalent with inactive Fc domain) to the C1q-coated ELISA plate is inhibited by solid-phase C1q on beads, but not by an equal amount of fluid-phase C1q. Data shown are representative for two independent experiments; bars indicate means and error bars indicate SDs. One-way ANOVA with Dunnett's multiple comparison test, compared to no competition; ns, not significant; *** $p < 0.001$; **** $p < 0.0001$. NHS, normal human serum.

Human anti-C1q mAbs recognize C1q bound to a range of its natural ligands

Next, we studied binding of the panel of anti-C1q mAbs to C1q bound to a number of its natural ligands, including IgG, IgM, and CRP, in ELISA (Figure 3A). Binding of anti-C1q mAbs to C1q was detected on all of these ligands, providing evidence that anti-C1q mAbs recognize C1q in its native ligand-bound conformation. To examine the interaction of anti-C1q mAbs with C1q in a physiologically relevant cellular environment, binding on IgG-opsonized cells and necrotic cells was assessed. Alemtuzumab, a therapeutic anti-CD52 mAb, was chosen for its potent ability to induce classical pathway complement activation. The anti-C1q mAbs indeed exclusively bound to alemtuzumab-opsonized PBMCs in the presence of C1q (Figure 3B). Apoptotic and dead cells are known to be bound by C1q, which improves clearance of these cells by phagocytosis [22]. Several anti-C1q mAbs showed binding to C1q on necrotic cells, especially the mAbs with the highest avidity in earlier ELISA experiments, i.e., 1F4, 1F5, and 4F5 (Figures 1D and 3C). On western blot, unheated C1q, was detected by all anti-C1q mAbs, in contrast to heat-treated C1q (both reduced and non-reduced) (Supplementary Figure 1). This indicates that the epitopes on C1q also become exposed in immobilized C1q in this context, but are destroyed upon heat denaturation.

Human anti-C1q autoantibodies target multiple epitopes on the collagen-like region of C1q

To investigate the C1q domains and epitopes that are targeted by these anti-C1q mAb, binding competition between mAbs was explored in ELISA (Figure 4A). While all anti-C1q mAbs display the expected self-inhibition, each mAb show different levels of inhibition with the other mAbs. Two distinct groups of mAbs could be discerned, competing with each other but not with mAbs from the other group. Both groups consist of mAbs from multiple donors, while each group contains at least one mAbs originating from donor 2, showcasing diversity of targeted epitopes within one individual. These data provide evidence for at least two different epitopes on C1q that are recognized by human anti-C1q autoantibodies.

The binding sites for our anti-C1q mAbs were compared with anti-C1q from SLE patients. To this end, antibodies from serum of three SLE patients (SLE A, B, and C) were purified on protein A, and competition for binding to C1q between mAbs and SLE antibodies was evaluated (Figure 4B). Competition patterns were clearly split between the two groups identified in mAb–mAb competition. Epitopes targeted by SLE A and SLE B overlap with

epitopes of mAbs 1F4, 1F5, 3C3, and 12F6, while SLE C antibodies compete with mAbs 2D2, 4C11, 4D2, 4E6, and 4F5. Each anti-C1q mAb was inhibited by the presence of at least one of the polyclonal SLE antibody mixes, showcasing that our anti-C1q mAbs target the same or similar epitopes as anti-C1q autoantibodies in SLE patients.

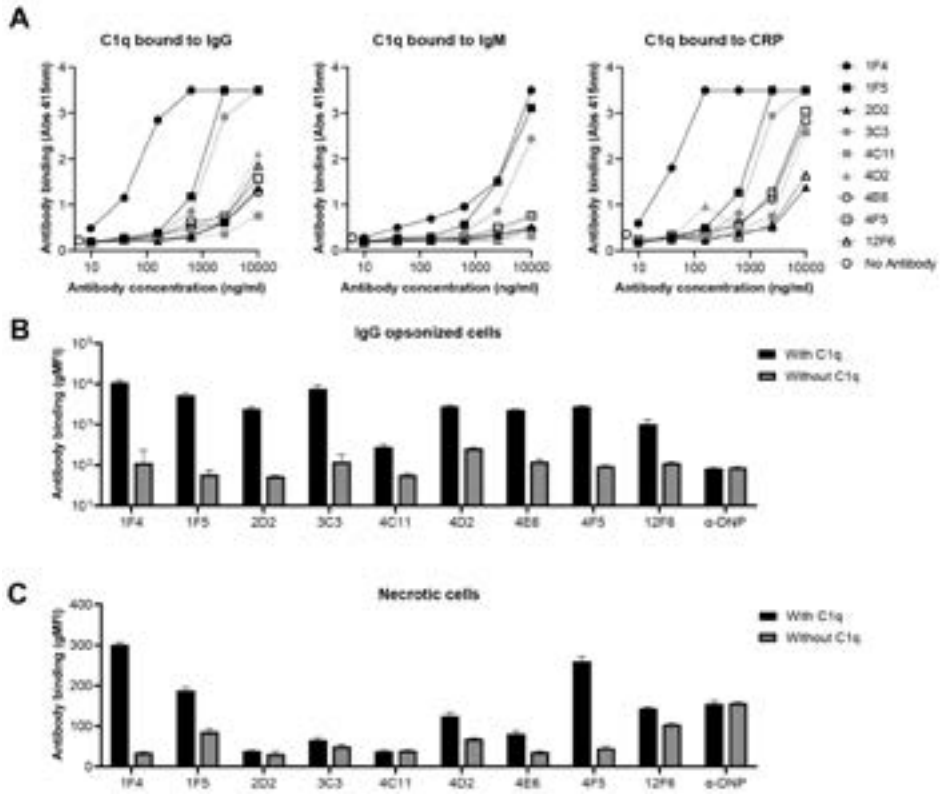


Figure 3. Binding of human anti-C1q mAbs to C1q on various ligands and surfaces. (A) Binding of anti-C1q mAbs to C1q on coated IVIG, IgM, and CRP in ELISA. (B) Binding of anti-C1q mAbs to alemtuzumab-opsonized PBMCs in flow cytometry, in the presence and absence of C1q. Anti-DNP was included as a negative control which does not bind C1q. (C) Binding of anti-C1q mAbs to necrotic PBMCs in flow cytometry, in the presence and absence of C1q. Anti-DNP was included as a negative control which does not bind C1q. All data shown are representative for at least two independent experiments. In panels (B and C), the geometric mean of fluorescence intensity (gMFI) and SD of triple measurements is indicated.

Additionally, a mixture of mAbs 1F4, 1F5, 4D2, and 4F5 showed near-complete inhibition of anti-C1q binding from purified SLE antibodies (Supplementary Figure 2). These data further indicate that in several anti-C1q-positive SLE patients, there is no anti-C1q reactivity present which is not covered by the set of anti-C1q mAbs presented in this manuscript.

A screening of linear peptides with a length of 21 amino acids, covering all three chains of the C1q protein, revealed that none of the anti-C1q mAbs recognized a linear peptide (Supplementary Figure 3). In order to map the binding of anti-C1q mAbs to the different regions of the C1q molecule, binding to CLR and recombinant GH domain was evaluated (Figure 4C). All anti-C1q mAbs evidently bind to the CLR and not the GH when tested in ELISA, with the exception of mAb 12F6, which only bound to full C1q.

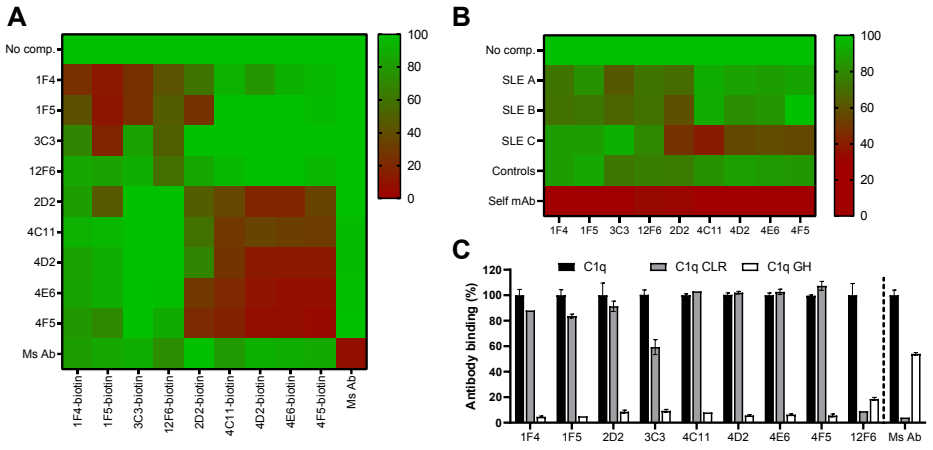


Figure 4. Determination of C1q epitope targeted by human anti-C1q mAbs. (A) Competition for binding to C1q-coated ELISA, between human anti-C1q mAbs, and with unrelated mouse anti-C1q mAb 4A4B11. The average percentage residual signal compared to no competition from two independent experiments is indicated in the heatmap. (B) Competition for binding to C1q-coated ELISA, between biotinylated anti-C1q mAbs and polyclonal anti-C1q antibodies from SLE serum, the percentage residual signal compared to no competition is indicated in the heatmap. Data are representative of two independent experiments. (C) Anti-C1q mAb binding to full C1q, C1q collagen-like region (C1q CLR), and C1q (recombinant) globular head (C1q GH) domains in ELISA; signal is normalized to 100% for binding to full C1q; mean and SD are shown. Ms Ab is mouse mAb 4A4B11; data shown are representative for three independent experiments.

Electron tomographic imaging of anti-C1q bound to Ig-C1q complexes

In order to visualize the binding of anti-C1q mAbs to immune complexes with C1q, we used electron tomography. For the electron microscopy studies, a monovalent, Fc-inactive variant of the anti-C1q mAb 1F5 was used to avoid C1q–Fc interaction and bivalent binding. First, anti-C1q mAb binding to C1q–(IgG)6 complexes was visualized using negative stain electron tomography (Figure 5A). C1q–(IgG)6 complexes could be directly interpreted in the tomographic slices. However, since the anti-C1q antibodies bound to more than one binding site per complex and the whole complex is highly flexible, it was not straightforward to visualize anti-C1q antibody molecules directly. As a metric to determine where anti-C1q IgG were located, we masked a circular region

of 26 nm, corresponding to the approximate diameter of the C1q–(IgG)6 complex, and any additional density bound to, but outside of, this region was interpreted as an anti-C1q mAb (Figure 5B). These data were used to build molecular models of the C1q–(IgG)6–anti-C1q complexes, which could be overlaid with the tomographic slices to aid interpretation (Figure 5C).

We observed multiple anti-C1q antibodies bound per complex, located at the periphery of the C1q–(IgG)6 complex. These observations demonstrate the presence of multiple epitopes of a monoclonal autoantibody on each C1q protein. Interestingly, although anti-C1q mAbs recognize an epitope in the CLR of C1q, we could observe that they bind in close proximity to the GH domains and not, as hypothesized beforehand, to the top stalk of the C1q molecule. These structural data provide important insights into the molecular interaction between C1q and C1q autoantibodies.

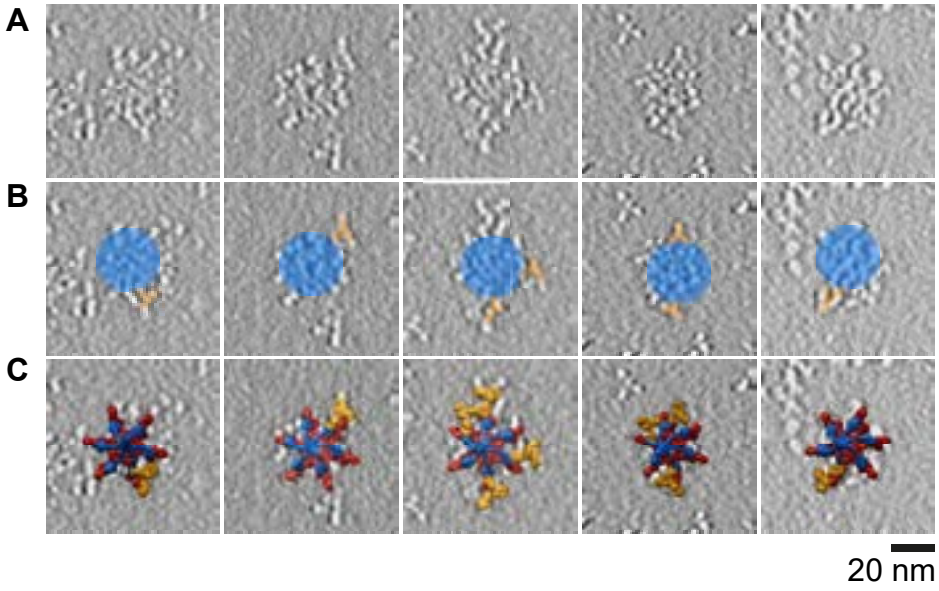


Figure 5. Electron tomography images of C1q bound by anti-C1q mAb. (A) 4.5-nm thick negative stain electron tomogram slices of hexameric anti-CD52 IgG-RGY, C1q, and monovalent, Fc-inactive anti-C1q mAb 1F5. (B) Overlay indicating the complex of hexameric IgG and C1q (blue area) and proposed location of anti-C1q mAb (yellow). (C) Overlay indicating the IgG (red), C1q (blue), and anti-C1q mAb (yellow) complex model on top of negative stain tomographic slices for better visualization (Scale bar, 20 nm).

Anti-C1q mAbs increase Fc-receptor engagement, but not complement activation, on immune complexes

We sought to understand the consequences of the presence of anti-C1q autoantibodies on the activation of the immune system by C1q-containing immune complexes. When anti-C1q binds to C1q on an immune complex, its Fc domain may add to the immune response by increasing complement activation or by Fc-receptor engagement and cell-mediated effector functions. Plate-bound IgG complexes allow classical pathway complement activation, in this case detected by C5b9 deposition with increasing serum concentration between 0.5% and 4% normal human serum (NHS) (Figure 6A). From this titration, we selected 1% NHS as a source of serum to further analyze whether the presence of anti-C1q mAbs would impact on the degree of complement activation. The presence of anti-C1q mAbs did not or only slightly increase complement activation, and in the case of two of the mAbs, a significant decrease of C5b9 generation was observed (Figure 6B).

When investigating Fc-receptor binding in the same context, deposited IgG complexes interacted with FcγRIIIa. Importantly, when C1q was present on the hexameric IgG, the binding of FcγRIIIa is highly impaired (Figure 6C). From the titration, we chose 3 µg/mL of FcγRIIIa to analyze the impact of anti-C1q mAbs. We observed that several anti-C1q mAbs were able to increase FcγRIIIa binding on C1q-containing immune complexes by 20% to 60% (Figure 6D). Interestingly, anti-C1q mAbs 3C3 and 4F5 show the highest FcγRIIIa binding, while also causing a decrease in complement activation of immune complexes. Focusing on functional consequences of Fc-receptor binding by anti-C1q mAbs, we investigated binding of IgM-coated beads opsonized with C1q by THP-1 cells differentiated toward macrophages. In this setting, the binding of IgM-coated/C1q opsonized beads is minimal, but addition of 1 µg/mL anti-C1q mAb increased binding of the beads to the differentiated THP-1 cells as much as fivefold (Figure 6E). When Fc receptors of cells are partially blocked by pre-incubating with an Fc receptor blocking agent, inhibition of binding of opsonized beads is observed, confirming that this is an Fc receptor–driven process (Figure 6F). In a similar fashion, we studied whether anti-C1q mAbs could enhance phagocytosis of bacteria by human neutrophils. *Staphylococcus aureus* was labeled with a monoclonal antibody targeting wall teichoic acid (WTA, an *S. aureus* surface glycopolymer [23]. Specifically, we used anti-WTA IgG4 because this isotype does not drive Fc-receptor-mediated phagocytosis directly but can interact with C1q after introduction of hexamer-enhancing mutations [24]. Anti-C1q mAbs were able to enhance phagocytosis of bacteria to various degrees after opsonization with an antibody isotype that does not facilitate phagocytosis (Figure 6G). Anti-C1q mAbs thus

do not notably enhance complement activation but highly enhance the capacity both IgM- and IgG-opsonized and C1q-containing immune complexes to engage Fc receptors and induce effector function.

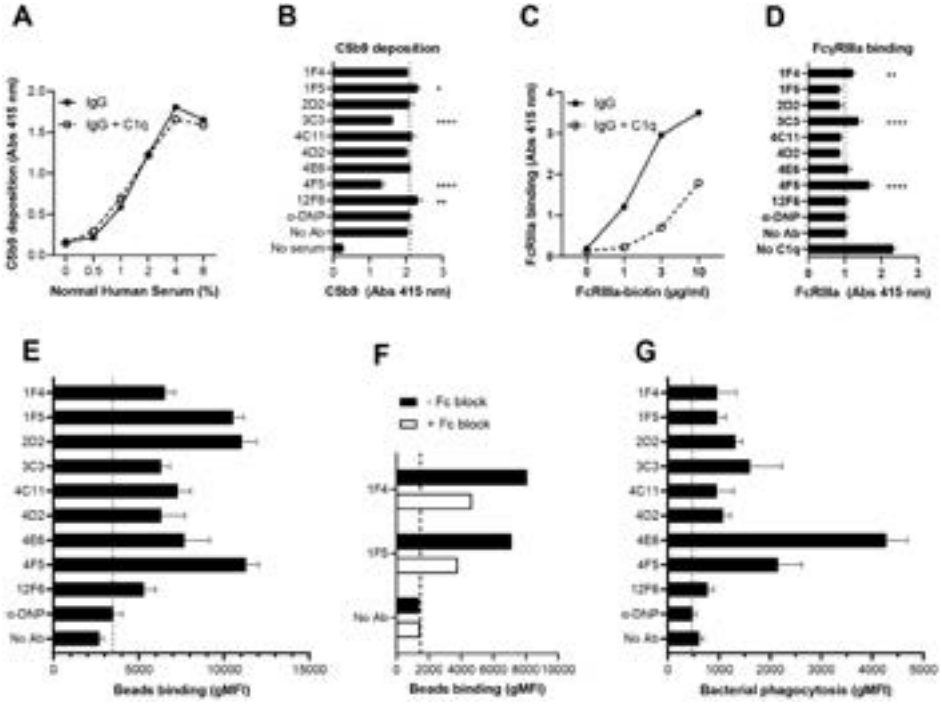


Figure 6. Anti-C1q mAbs stimulate Fc receptor engagement and phagocytosis, but not complement activation. (A) Complement activation on hexameric IgG complexes with or without extra C1q in ELISA was detected on the level of C5b9. On the basis of the NHS titration, we selected 1% NHS for the following experiment to investigate the effect of anti-C1q mAbs. (B) C5b9 deposition on hexameric IgG complexes in ELISA, in the presence of different anti-C1q mAbs at 1% NHS. (C) Binding of biotinylated FcγRIIIa to hexameric IgG complexes with or without extra C1q in ELISA, on the basis of the FcγRIIIa titration, we used 3 µg/mL FcγRIIIa in the following experiment to investigate the effect of anti-C1q mAbs. (D) FcγRIIIa-biotin binds to hexameric IgG complexes and different anti-C1q mAbs in ELISA. (E) PMA-differentiated THP-1 cells bind IgM-coated/C1q-opsonized beads, in the presence of anti-C1q mAbs. (F) PMA-differentiated THP-1 cell interaction with IgM-coated/C1q-opsonized beads is inhibited by an Fc-blocking agent. (G) Human neutrophils phagocytose Staphylococcus aureus bacteria opsonized with anti-WTA IgG4-E430G and C1q, in the presence of anti-C1q mAbs. For B and D–F, mean and SD are shown; data are representative for three experiments. For (B and D), each anti-C1q mAb was compared to anti-DNP with one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Discussion

In the current study, we investigated the characteristics and functional properties of human anti-C1q autoantibodies on a monoclonal level. Autoantibodies against C1q are present in several diseases such as SLE and HUVS but also in a substantial number of healthy individuals [5, 6]. In SLE, the presence of anti-C1q autoantibodies is associated with the development of lupus nephritis [9]. Experimental studies indicate that anti-C1q autoantibodies contribute to renal disease only when there are C1q-containing immune complexes in the glomeruli [7]. In this study, we have obtained a set of anti-C1q mAbs from healthy individuals and confirmed that these antibodies have a similar binding profile as anti-C1q autoantibodies in SLE patients. The information obtained from the analysis of the molecular properties of these anti-C1q autoantibodies sheds light on a role of anti-C1q in healthy individuals as well as on a role for these antibodies in pathological conditions such as lupus nephritis. Importantly, the molecular properties of these anti-C1q autoantibodies may allow the development of specific therapeutic or diagnostic tools.

We have obtained nine anti-C1q mAb, all derived from healthy individuals. Our attempts to isolate anti-C1q-producing B cell clones from SLE patients were not successful, possibly due to the immunosuppressive treatment that the patients received. To confirm that the analysis of anti-C1q mAbs isolated from healthy donors is meaningful for the understanding of anti-C1q antibodies in SLE, we performed competition experiments using purified IgG of anti-C1q-positive SLE patients. This confirmed that the anti-C1q mAbs isolated from healthy donors indeed all target C1q epitopes that are also targeted by anti-C1q autoantibodies present in SLE patients, underscoring the relevance of the identified anti-C1q mAbs. By isolating only anti-C1q of the IgG isotype and producing it recombinantly in IgG1 subclass, some information on the presence of various isotypes and subclasses may have been lost. However, IgG is evidently the dominant isotype among anti-C1q autoantibodies in SLE patients, while the subclasses IgG1, IgG2, and IgG3 are all regularly found [25, 26].

All anti-C1q clones were first identified using ELISA with anti-IgG detection and amplified with primers specific for IgG. Therefore, the B cells producing these anti-C1q in vivo must have undergone class-switching to IgG. In B cells, class-switching and avidity maturation often occur at the same developmental stage. Indeed, we observed high avidity of anti-C1q for its antigen in SPR experiments. The range of binding strengths observed in ELISA and cellular assays was not fully mirrored in the SPR results, where less variation in avidity was detected. These differences may be explained by the different manner of presenting solid-phase C1q or by the fundamental difference in techniques, as ELISA requires interactions to resist multiple washing and incubation steps to be registered,

whereas SPR does not and consequently measures more native-like interactions. These steps in ELISA and cellular assays may work to amplify any differences in binding strength [27]. Interestingly, class-switching and avidity maturation are both processes that only commence in B cells if there is sufficient T cell help, indicating that in SLE patients and in healthy controls, T cell help for C1q-reactive B cells must be present. Currently, there are no studies into the nature of the T cell help. This would be highly interesting as the B cells are reactive to a conformational epitope and the T cells will be reactive to a linear peptide presented in human leukocyte antigen (HLA); this may be a C1q peptide or a peptide from any protein in the C1q-containing immune complex.

In determining the epitope targeted by anti-C1q mAbs, we could classify our anti-C1q mAbs in two groups based on competition assays. All but one anti-C1q mAb bound to C1q CLR almost as strong as to full C1q. The binding to C1q CLR is in accordance with earlier studies on anti-C1q-antibody-positive sera [28]. The presented slices through electron tomographic volumes indicate binding of anti-C1q mAb 1F5 to an epitope on the CLR close to the GH domain of C1q. While no exact epitope could be determined, this experiment contributes to identifying one of the regions of C1q targeted by human anti-C1q autoantibodies. The location of antibody binding and the observation that multiple anti-C1q 1F5 mAbs can bind to one C1q molecule also indicate that the target epitope is likely present on the extended arms of the C1q CLR and not on the central CLR stalk where all arms come together. This may be explained by the GH domains bending at the interface of CLR and GH upon docking onto a ligand [29, 30], revealing a cryptic binding site previously obscured when in fluid phase. Collectively, the data indicate that anti-C1q mAbs bind C1q in a conformationally changed state, which occurs in C1q following binding to its natural ligands, or to surfaces (Figure 7 A–C).

While the origin of anti-C1q autoantibody reactivity is still unclear, there may be some benefit to the host under certain conditions, once these antibodies have arisen. We hypothesized that anti-C1q antibodies of the IgG isotype could aid in phagocytosis of C1q-opsonized particles by engaging Fc receptors. Indeed, addition of anti-C1q mAbs increased binding and phagocytosis of opsonized beads and *S. aureus* bacteria. In vivo, the same mechanism could enhance the clearance of C1q-opsonized pathogens and apoptotic cells.

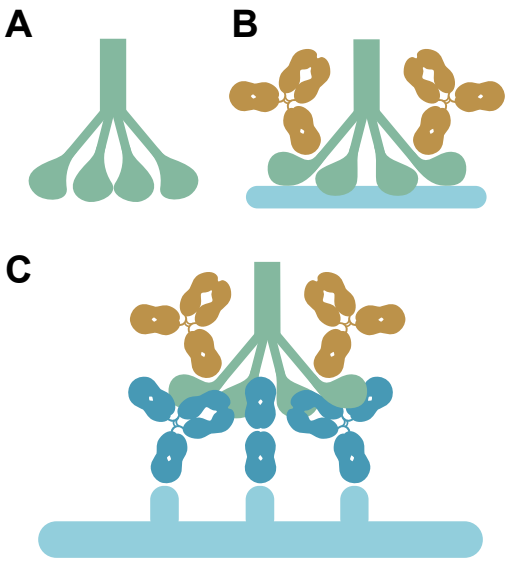


Figure 7. Schematic overview of binding of anti-C1q to solid-phase C1q. (A) Fluid-phase C1q is not bound by human anti-C1q autoantibodies. (B and C) When C1q binds to a (cell) surface (B) or ligand such as IgG complex (C), it adopts a solid-phase conformation. This conformation reveals a previously hidden epitope, targeted by anti-C1q autoantibodies, allowing them to specifically bind to solid-phase C1q.

Nonetheless, the presence of anti-C1q autoantibodies in SLE patients is heavily linked to nephritis [7-9], likely because of the additional immune activation on C1q-containing immune complexes deposited in the glomeruli. In a mouse model of anti-C1q enhancement of immune-complex nephritis, we observed earlier that both complement activation and Fc receptor engagement were necessary for kidney damage [7]. Based on the current findings, we now hypothesize that complement activation by the immune complexes in the kidney is required to attract inflammatory cells to the glomerulus but is not enhanced by anti-C1q autoantibodies. The anti-C1q driven Fc-receptor triggering would then mediate damage to the kidneys by the newly attracted immune cells. Future research using in vivo experiments in mice would be needed to further support this hypothesis. Unfortunately, such an experiment is currently not possible, as these human anti-C1q mAbs bind strongly to human C1q, but do not bind substantially to mouse C1q.

In our experiments, anti-C1q mAb binding to solid-phase C1q was not, or only marginally, inhibited by fluid-phase C1q. Reversely, anti-C1q mAb binding could be inhibited in the same assay with solid-phase C1q on beads, re-iterating the specificity of these antibodies. The specific targeting of solid-phase C1q could potentially be used

diagnostically or therapeutically. In a diagnostic setting, the isolated anti-C1q mAbs may be used to identify tissue locations where C1q is activated in vivo. Therapeutically, anti-C1q mAbs may be exploited in approaches to enhance or decrease C1q-immune complex-mediated effects.

The current study reveals molecular properties of human anti-C1q autoantibodies on a monoclonal level. These autoantibodies bind specifically to solid-phase C1q, which exposes cryptic epitopes not available in fluid-phase C1q. The data provide insights into the immunopathological processes that underlie lupus nephritis and may therefore be an important step in fighting this autoimmune disease.

Materials and methods

ELISA to screen individuals for anti-C1q-antibody positivity

Serum of selected individuals was screened for the presence of anti-C1q antibodies by QUANTA Lite Anti-C1q (Werfen) enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Werfen). Briefly, samples were diluted 1:101 in Sample Diluent and 100 µL diluted samples or supplied controls were added to the wells. After 30-min incubation, wells were washed and incubated with 100 µL horseradish peroxidase (HRP) IgG conjugate for another 30 min. Next, wells were washed and stained for 30 min with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) Chromogen while protected from light; then, 100 µL HRP Stop Solution was added. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark) and used to calculate anti-C1q units based on positive control samples provided in the kit. The cut-off for positivity was 20 units, as recommended by the manufacturer.

Isolation of C1q-reactive B cells

To optimize the anti-C1q staining procedure, HEK cells were transduced to express mouse anti-C1q mAb JL-1 on the cell surface, as described before [7, 31]. The resulting cells mimic B cells with a C1q-binding B cell receptor. For fluorescence-activated cell sorting (FACS) of B cells, peripheral blood was collected from anti-C1q-positive donors after obtaining their informed consent, approval was granted by the Medical Ethical Committee of Leiden-The Hague-Delft (reference numbers: B19.008/AB/ab and P17.151). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (Leiden University Medical Center [LUMC] pharmacy) gradient centrifugation and B cells were enriched using the EasySep Human B cell isolation kit (Stem Cell Technologies) following instructions provided by the manufacturer. Fluorescent solid-phase C1q-containing complexes were

generated by mixing purified C1q (Complement Technologies), pre-formed hexamers of anti-dinitrophenol (DNP) IgG1-RGY antibodies, biotinylated peptides containing DNP (LUMC peptide facility) and streptavidin-phycoerythrin (PE) (Bio Rad) or streptavidin-AlexaFluor647 (Invitrogen) (Figure 1B). IgG1 antibodies (mAb G2a-2) directed against the hapten DNP [32] were engineered with hexamerization-enhancing mutations E345R, E430G, and S440Y to generate hexamers of IgG that bind C1q, thereby bringing it in its solid-phase conformation [30, 33]. The anti-DNP antibodies bind a peptide containing DNP and biotin, linking it to the streptavidin-coupled fluorochromes.

B cells were incubated with fluorescent solid-phase C1q complexes for 45 min at 4 °C. After washing in phosphate-buffered saline (PBS) with 1% fetal calf serum (FCS), the cells were further stained with mouse anti-human CD27-FITC (ThermoFisher; mAb CLB-27/1), CD3-Pacific Blue (BD Biosciences; mAb SP34-2) and IgD-PE-Cy7 (BD Biosciences, mAb IA6-2) and incubated for 45 min at 4 °C [34]. Single B cells detected as $CD3^-$, IgD^-, $CD27^+$, and double positive for C1q-complex staining were sorted on a FACSAria III Cell Sorter (BD Biosciences) and collected at one cell per well in a 96-well flat bottom plate (Corning). The wells contained 100,000 irradiated (50 Gy) EL4B5 cells expressing CD40L in 200 µL Iscove's Modified Dulbecco's Medium (IMDM; Lonza) supplemented with 10% FCS, 2 mM L-glutamine (Gibco), 50 µM 2-mercaptoethanol (Sigma-Aldrich), 100 units/mL penicillin, 100 µg/mL streptomycin (both Gibco), 20 µg/mL Insulin-transferrin-sodium selenite (Sigma-Aldrich), 50 ng/mL interleukin (IL)-21 (Gibco), 1 ng/mL IL-1β (Miltenyi Biotec), 0.3 ng/mL tumor necrosis factor (TNF)-α (Miltenyi Biotec), and 0.5 µg/mL Resiquimod (R848; Sigma-Aldrich) [35, 36]. Plates with B cells were incubated for 13 d at 37 °C and 5% CO_2 before analysis.

Identification and variable domain sequence analysis of anti-C1q-producing B cells

Supernatant of sorted B cells was harvested after a 13-d expansion period and screened for IgG production by ELISA as described before [37]. Screening for C1q reactivity in the B cell supernatant was performed by coating C1q instead of goat anti-human-IgG. B cell clones which were positive for anti-C1q production were lysed and RNA was isolated using TRIzol reagent (Invitrogen). Subsequently, cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara) followed by rapid amplification of cDNA ends (RACE) PCR to amplify variable domains of the heavy (VH) and kappa or lambda light (VL) chain. Isolated VH and VL fragments were ligase-independently cloned into pcDNA3.3 plasmids containing IgG1, kappa or lambda constant domains, as described before [38]. The plasmids were then sequenced by the Leiden Genome Technology

Center to obtain the VH and VL sequences. Sequences were analyzed for VDJ gene usage and complementarity-determining region (CDR) identification by IMGT V-quest [39].

Production and purification of monoclonal antibodies

Heavy and light chain plasmids were co-transfected in Expi293F cells using ExpiFectamine, Opti-MEM, and Expi293 expression medium (all ThermoFisher) according to the manufacturer's instructions. Supernatant was harvested after 5–7 d and filtered, and antibodies were purified on protein A resin (Genscript). Subsequently, buffer was exchanged to PBS, and antibodies were concentrated on 50 kDa Amicon centrifugal filters (Merck Millipore). Antibody concentration was measured using an in-house sandwich ELISA as described previously [37].

ELISA for binding of anti-C1q mAbs to C1q on natural ligands and polyreactivity

ELISA was performed to evaluate reactivity of the isolated mAbs toward C1q on natural ligands and toward common targets of polyreactive antibodies. Nunc MaxiSorp plates (ThermoFisher) were coated with 10 µg/mL calf thymus single-stranded DNA (Sigma-Aldrich), lipopolysaccharide (LPS; Sigma-Aldrich), pre-pro-insulin (produced in-house), intravenous immunoglobulins (IVIG; Sanquin), CRP (Calbiochem), 5 µg/mL IgM (Sigma-Aldrich) or 5 µg/mL C1q in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$, at pH 9.6) for 1 h at 37 °C. Plates were washed three times with PBS/0.05% Tween after every incubation. Plates were blocked with 100 µL/well PBS/1% bovine serum albumin (BSA) for 1 h at 37 °C. Wells coated with IVIG, IgM, or CRP were incubated with 5 µg/mL C1q diluted in PBS/0.05% Tween/1% BSA (hereafter abbreviated to PTB) for 1 h at 37 °C. After washing, plates were incubated with anti-C1q mAbs biotinylated with the Pierce Antibody Biotinylation Kit (ThermoFisher) diluted in PTB and incubated for 1 h at 37 °C. Binding of anti-C1q mAbs was detected by 0.5 µg/mL HRP-coupled streptavidin (ThermoFisher) in PTB, incubated for 1 h at 37 °C. After the final washing sequence, 50 µL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/0.015% H_2O_2 (both from Merck) was added, and absorbance at 415 nm was measured using a microplate reader.

Avidity measurement of anti-C1q mAbs by surface plasmon resonance

Surface plasmon resonance was employed to determine the avidity of anti-C1q mAbs for C1q, using a Biacore T200 (Cytiva). Biotinylated C1q was immobilized on a streptavidin-

coated chip (Cytiva) to a response of 146.4 response units, with an empty channel for compensation. By immobilizing C1q on the chip, it was presented in solid-phase. Next, titration curves of anti-C1q mAbs or unrelated mouse anti-C1q mAb 4A4B11 (ATCC HB-8327) in PBS/0.05% Tween/0.5 mg/mL BSA from 40 to 0.05 µg/mL were prepared. These samples were stored in the Biacore at 4 °C and covered with a breakable seal during the measurement to ensure a stable quality of the samples. Samples were run in increasing concentration steps, with chip regeneration by two times 30 s flowing 10 mM glycine, pH = 2.0. Resulting binding curves were analyzed using Biacore T200 Evaluation Software 3.2.1 (Cytiva) to fit a 1:1 binding model, resulting in association and dissociation constants for each anti-C1q mAbs.

ELISA to determine inhibition of anti-C1q mAbs by fluid-phase C1q

ELISA plates were coated with 10 µg/mL C1q in coating buffer overnight at 4 °C and subsequently blocked with PBS/1%BSA for 1 h at 37 °C. Biotinylated anti-C1q mAbs were mixed with either 20 µg/mL C1q, 20 µg/mL C1 (Complement Technologies), 5% or 25% pooled normal human serum (NHS) or 5% or 25% C1q-depleted serum (Complement Technologies), in PTB containing 0.5 M NaCl to prevent C1q–Fc interactions [13]. Mouse mAb 4A4B11 was tested as a control which is not specific for solid-phase C1q. Samples with serum were also supplemented with 10 mM EDTA to prevent complement activation. After pre-incubation on ice for 30 min, samples were incubated in the C1q-coated wells for 1 h at 37 °C. Anti-C1q mAbs were detected with 0.5 µg/mL streptavidin-HRP, plates were then developed by incubating with ABTS/0.015% H_2O_2 and measured using a microplate reader. Percentage signal was calculated by setting the absorbance values of samples without C1q competition at 100% for each individual mAb.

ELISA to determine inhibition of monovalent anti-C1q mAbs by solid-phase C1q

For mAbs 1F4, 1F5, and 4F5, antibody was produced containing LALA-PG mutations to render their Fc domain incapable of recruiting C1q [LALA-PG and F405L or K409R mutations to allow for Fab-arm exchange. These antibodies were then combined with the anti-HIV gp120 clone b12 through Fab-arm exchange following the protocol described by Labrijn *et al.* [40, 41]. The resulting bispecific antibodies (bsAb) were functionally monovalent for C1q binding, with inactive Fc domains to specifically investigate the interactions between one C1q molecule and one antigen binding domain. ELISA plates were coated with 10 µg/mL C1q in coating buffer overnight at 4 °C and subsequently blocked with PBS/1%BSA for 1 h at 37 °C. Biotinylated anti-C1q bsAb in PTB were mixed

with C1q or human serum albumin (HSA) coupled to Carboxyl Fluorescent Particles, 0.4–0.6 µm (Spherotech), or soluble C1q or HSA. After pre-incubating for 30 min, each well in the ELISA plate received 0.1 µg anti-C1q antibody and 1 µg C1q or HSA, which in case of solid-phase competition was coupled to 10^9 beads. After incubating for 1 h at 37 °C, plates were washed and bound, biotin-labeled anti-C1q antibodies were detected with 0.5 µg/mL streptavidin-HRP. Plates were developed by incubating with ABTS/0.015% H_2O_2 and measured using a microplate reader.

Anti-C1q mAbs binding to C1q on opsonized and necrotic cells

PBMCs were isolated from healthy donor peripheral blood by Ficoll-Paque gradient centrifugation. Cells were either opsonized with recombinant anti-CD52 (alemtuzumab) IgG1 antibodies containing hexamer-enhancing mutations RGY for 45 min at 4 °C or made necrotic by incubating for 30 min at 56 °C [42, 43]. Cells were then seeded into 96-well plates at 100,000 cells per well and washed two times by adding 200 µL FACS buffer (PBS/2% FCS), centrifuging and removing the supernatant. C1q was added at 2 µg/mL in FACS buffer and incubated with the cells for 45 min at 4 °C. After washing, cells were incubated with 10 µg/mL biotinylated anti-C1q mAbs for 45 min at 4 °C and washed again. Binding of anti-C1q mAbs to the cells was detected by incubating with 2 µg/mL streptavidin-AlexaFluor647 for 45 min at 4 °C. As positive control for C1q binding, polyclonal rabbit anti-C1q (DAKO) and BrilliantViolet421-labeled donkey anti-rabbit IgG (BioLegend) were used. After the final wash, the fluorescent signal on the cells was measured on a FACSCanto flow cytometer (BD Biosciences).

ELISA for competition between anti-C1q mAbs and purified SLE antibodies

ELISA plates were coated with 10 µg/mL C1q in coating buffer overnight at 4 °C and subsequently blocked with PBS/1%BSA for 1 h at 37 °C. Competitor anti-C1q mAb at 64 µg/mL final concentration, or protein A-purified SLE antibodies at 5 mg/mL, was added to the plates in PTB and incubated 1 h at 37 °C. Without washing, biotinylated anti-C1q mAb was then added at concentration between 0.15 and 12 µg/mL depending on the concentration needed to obtain near saturation binding signal without competition. After incubation for 1 h at 37 °C, plates were washed and biotinylated anti-C1q mAb binding was detected by 0.1 µg/mL streptavidin-HRP. Plates were developed by incubating with ABTS/0.015% H_2O_2, and absorbance was measured at 415 nm using a microplate reader. Percentage residual binding was calculated by dividing absorbance in presence of competition by absorbance in the absence of competition.

ELISA to detect binding of anti-C1q mAbs to C1q collagen-like region and globular heads

ELISA plates were coated with C1q CLR made by limited proteolysis of purified serum C1q, recombinant single-chain GH domains (described in ref. [44]) (both gifts from Nicole Thielens), or purified intact C1q at 10 µg/mL in coating buffer for 1 h at 37 °C. The plates were then blocked with PBS/1%BSA for 1 h at 37 °C, washed, and incubated with 4 µg/mL anti-C1q mAb, or 10 µg/mL for mAb 12F6, for 1 h at 37 °C. After washing, bound anti-C1q mAb was detected with 1:2000 rabbit anti-human IgG-HRP, plates were developed by incubating with ABTS/0.015% H_2O_2, and absorbance at 415 nm was measured using a microplate reader. Absorbance values for binding to intact C1q were set to 100% for each mAb to facilitate easier comparison.

Sample preparation and data collection for negative stain electron tomography

C1q (270 µg/mL final concentration) was incubated with IgG1-anti-CD52-RGY (540 µg/mL final concentration) for 30 min at 4 °C. Monovalent, Fc-inactive anti-C1q bsAb combining 1F5 with control b12 (made as described for the solid-phase C1q inhibition ELISA; used at 90 µg/mL final concentration) was added and samples were incubated for another 30 min at 4 °C. Samples were purified using a Superdex 200 Increase 3.2/300 column (Cytiva). Column was equilibrated with PBS on an Äkta pure system (Cytiva). Size exclusion fractions were diluted 1:10 in water and loaded on freshly plasma-cleaned 200 mesh carbon-coated copper grids (Electron Microscopy Sciences) and incubated for 1 min, before blotting using Whatman paper. Samples were stained using 2% uranyl formate for 1 min. Negative stain tilt-series were collected on a FEI Tecnai T12 Biotwin with LaB6 source, operating at 120 kV on a FEI Eagle 4 k × 4 k CCD camera. Tilt series were collected using Xplore 3D (ThermoFisher Scientific) at 49,000× magnification and a pixel size of 4.546 Å using a continuous acquisition scheme from ±60° with a tilt increment of 3°. A total dose of 100 e^-/Å2 and a defocus of −4 µm was used. Tracking and focusing were performed before every third image acquisition.

Tomogram reconstruction

Alignment of cryo-electron tomography raw frames was performed using the "alignframes" command from the software program IMOD 4.11.13 [45]. Additionally, IMOD was used to reconstruct negative stain tomograms using weighed back projected with a SIRT-like filter equivalent to five iterations. Tomograms were aligned using patch tracking. IgG1–C1q–1F5 maps were displayed on the tomographic slices using UCSF Chimera 1.16 [46].

ELISA for complement activation in the presence of anti-C1q mAbs

IgG1 anti-DNP with RGY mutations for hexamer formation were coated on ELISA plates at 10 µg/mL in coating buffer for 1 h at 37 °C. The plates were blocked with PBS/1%BSA for 1 h at 37 °C, washed and incubated with 10 µg/mL C1q for 1 h at 37 °C. After washing, 50 µg/mL anti-C1q mAb was incubated in the wells for 1 h at 37 °C. The wells were washed, incubated with 1% NHS in RPMI 1640 (Gibco) for 1 h at 37 °C, and washed again. Deposited C5b-9 was detected using 333x diluted Mouse anti-C5b9 (clone aE11, DAKO) and Goat anti-Mouse-HRP (DAKO). Plates were developed with ABTS/0.015% H_2O_2 and absorbance at 415 nm was measured using a microplate reader.

ELISA for FcγRIIIa binding in presence of anti-C1q mAbs

FcγRIIIa with a C-terminal 10xHis and BirA tags were produced in Freestyle 293-F cells (ThermoFisher), purified on a His-trap column (GE Life Sciences) and biotinylated with BirA as described previously [47]. IgG1 anti-DNP with RGY mutations for hexamer formation were coated on ELISA plates at 10 µg/mL in coating buffer for 1 h at 37 °C. The plates were blocked with PBS/1%BSA for 1 h at 37 °C, washed and incubated with 10 µg/mL C1q for 1 h at 37 °C. After washing, 50 µg/mL anti-C1q mAb was incubated in the wells for 1 h at 37 °C. The wells were washed and then incubated with 3 µg/mL FcγRIIIa-biotin for 1 h at 37 °C. Detection of FcγRIIIa-biotin after washing was performed by incubating with 0.1 µg/mL streptavidin-HRP for 1 h at 37 °C. After adding ABTS/0.015% H_2O_2, absorbance at 415 nm was measured using a microplate reader.

Binding of anti-C1q opsonized beads by THP-1 cells

The THP-1 cell line (ATCC TIB-202) was cultured in RPMI (Gibco) with 10% FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (all Gibco). Cells were differentiated to a macrophage phenotype by incubating 100,000 cells/well in a 48-well plate with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) in the medium. After 3 d incubating at 37 °C, medium was replaced with PMA-free medium and cells were incubated for another 5 d. Yellow fluorescent 0.46-µm carboxyl beads (Spherotech) were loaded with IgM for 1 h at 37 °C, washed three times in PBS, and incubated with C1q for 1 h at 4 °C. After washing, beads suspension was incubated with anti-C1q mAb in serum-free medium for 1 h at 4 °C, after which the mixture was added to the differentiated THP-1 cells. Per well, 3 × 108 beads, incubated with 0.1 µg IgM, 0.5 µg C1q, and 0.2 µg anti-C1q mAb were added in a total volume of 200 µL serum-free medium. For experiments with Fc blocking reagent, cells were incubated with 10 µg/mL Human Fc block (BD Biosciences) for 10 min at room temperature before adding

the beads suspension. Plates were centrifuged for 1 min at 100g to bring the beads in contact with the cells and were then incubated for 1 h at 37 °C to allow binding and phagocytosis of anti-C1q mAb-covered beads by the cells. The beads suspension was removed, and the cells were treated with trypsin (Gibco) to detach them from the plate. Cells were resuspended, washed in FACS buffer and then analyzed on a FACSCanto flow cytometer. Binding or phagocytosis was measured by FITC fluorescence. Differentiation of THP-1 cells was confirmed by increased expression of CD11b (by mouse anti-CD11b-APC, clone D12, BD Biosciences) and decreased expression of CD15 (by mouse anti-CD15-BV510, clone W6D3, BD Biosciences).

Phagocytosis of S. aureus by human neutrophils

Human polymorphonuclear (PMN) leukocytes were isolated freshly from blood of healthy donors by the Ficoll-Histopaque gradient method [48]. In 96-well plates, mAmetrine-labeled *S. aureus* (strain Newman∆spa/sbi; 750,000 cells/well) was mixed with 1.5 µg/mL mAb [IgG4 with E430G mutation against wall teichoic acid (WTA) and 6 µg/mL C1q in RPMI supplemented with 0.05% HAS [24, 49]. Plates were incubated for 15 min at 37 °C on an orbital shaker. Subsequently, anti-C1q mAbs were added at 10 µg/mL, and plates were incubated for another 15 min at 37 °C on an orbital shaker. Finally, PMN leukocyte cells were added at 75,000 cells/well to allow phagocytosis of opsonized bacteria. After 15-min incubation at 37 °C, phagocytosis was stopped by addition of 1% paraformaldehyde. Neutrophils were gated based on forward and sideward scatter, and the fluorescence of mAmetrine-labeled bacteria associated with neutrophils was acquired.

Statistical analysis

Where applicable, statistical analysis of results was performed in GraphPad Prism software version 9.3. EC50 of anti-C1q mAb binding to C1q-coated ELISA was determined from an agonist vs. response curve with variable slope (four parameters). Significance for solid-phase C1q inhibition, complement activation, and FcγRIIIa binding was determined per anti-C1q mAb by one-way ANOVA followed by Dunnett's multiple comparisons test. A P-value below 0.05 was considered statistically significant.

Acknowledgements

We thank the patients, donors, doctors, and other technical staff in the Leiden University Medical Center (LUMC) for providing the samples. We are grateful to Nicole Thielens

for supplying C1q CLR and GH domains. We thank Maartje Ruyken (University Medical Center Utrecht) for assistance in phagocytosis experiments. Furthermore, we thank the Flow Cytometry Core Facility of the LUMC for their help in cell sorting and flow cytometry experiments and Joost Bakker for aid in visualization. The project received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 101001937, ERC-ACCENT) to S.H.M.R., and from the LUMC Research profile: Immunity, Infection and Tolerance to L.A.T.

Author contributions

D.J.D., F.S.v.d.B., T.H.S., P.W.H.I.P., and L.A.T. designed research; D.J.D., L.A., R.Z., J.P., and L.d.V. performed research; C.S.M.K., L.M.S., J.W.D., K.A.G., A.Z., and G.V. contributed new reagents/analytic tools; D.J.D., F.S.v.d.B., L.A., L.d.V., T.H.S., and L.A.T. analyzed data; and D.J.D., F.S.v.d.B., L.A., C.S.M.K., L.M.S., S.H.M.R., G.V., T.H.S., P.W.H.I.P., and L.A.T. wrote the paper.

Competing interests

D.J.D. and L.A.T. are coinventors on a patent application describing antibodies against complement protein C1q.

References

1. D. Ricklin, G. Hajishengallis, K. Yang, J. D. Lambris, Complement: A key system for immune surveillance and homeostasis. *Nature immunology* **11**, 785 (2010).
2. R. A. van Schaarenburg, L. Schejbel, L. Truedsson, R. Topaloglu, S. M. Al-Mayouf, A. Riordan, A. Simon, M. Kallel-Sellami, P. D. Arkwright, A. Ahlin, . . . L. A. Trouw, Marked variability in clinical presentation and outcome of patients with c1q immunodeficiency. *J Autoimmun* **62**, 39-44 (2015).
3. D. J. Dijkstra, J. V. Joeloemsingh, I. M. Bajema, L. A. Trouw, Complement activation and regulation in rheumatic disease. *Semin Immunol* **45**, 101339 (2019).
4. M. A. Dragon-Durey, C. Blanc, M. C. Marinozzi, R. A. van Schaarenburg, L. A. Trouw, Autoantibodies against complement components and functional consequences. *Mol Immunol* **56**, 213-221 (2013).
5. F. S. van de Bovenkamp, D. J. Dijkstra, C. van Kooten, K. A. Gelderman, L. A. Trouw, Circulating c1q levels in health and disease, more than just a biomarker. *Mol Immunol* **140**, 206-216 (2021).
6. M. A. Seelen, L. A. Trouw, M. R. Daha, Diagnostic and prognostic significance of anti-c1q antibodies in systemic lupus erythematosus. *Curr Opin Nephrol Hypertens* **12**, 619-624 (2003).
7. L. A. Trouw, T. W. Groeneveld, M. A. Seelen, J. M. Duijs, I. M. Bajema, F. A. Prins, U. Kishore, D. J. Salant, J. S. Verbeek, C. van Kooten, M. R. Daha, Anti-c1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular c1q-containing immune complexes. *J Clin Invest* **114**, 679-688 (2004).
8. I. E. Coremans, P. E. Spronk, H. Bootsma, M. R. Daha, E. A. van der Voort, L. Kater, F. C. Breedveld, C. G. Kallenberg, Changes in antibodies to c1q predict renal relapses in systemic lupus erythematosus. *Am J Kidney Dis* **26**, 595-601 (1995).
9. M. Trendelenburg, J. Marfurt, I. Gerber, A. Tyndall, J. A. Schifferli, Lack of occurrence of severe lupus nephritis among anti-c1q autoantibody-negative patients. *Arthritis Rheum* **42**, 187-188 (1999).
10. D. Saadoun, S. Sadallah, M. Trendelenburg, N. Limal, D. Sene, J. C. Piette, J. A. Schifferli, P. Cacoub, Anti-c1q antibodies in hepatitis c virus infection. *Clin Exp Immunol* **145**, 308-312 (2006).
11. C. Magro-Checa, R. Schaarenburg, H. Beaart, T. Huizinga, G. Steup-Beekman, L. Trouw, Complement levels and anti-c1q autoantibodies in patients with neuropsychiatric systemic lupus erythematosus. *Lupus* **25**, 878-888 (2016).
12. F. J. Beurskens, R. A. van Schaarenburg, L. A. Trouw, C1q, antibodies and anti-c1q autoantibodies. *Molecular immunology* **68**, 6-13 (2015).
13. J. Kohro-Kawata, M. H. Wener, M. Mannik, The effect of high salt concentration on detection of serum immune complexes and autoantibodies to c1q in patients with systemic lupus erythematosus. *J Rheumatol* **29**, 84-89 (2002).
14. S. Uwatoko, S. Aotsuka, M. Okawa, Y. Egusa, R. Yokohari, C. Aizawa, K. Suzuki, C1q solid-phase radioimmunoassay: Evidence for detection of antibody directed against the collagen-like region of c1q in sera from patients with systemic lupus erythematosus. *Clin Exp Immunol* **69**, 98-106 (1987).

15. M. D. Golan, R. Burger, M. Loos, Conformational changes in c1q after binding to immune complexes: Detection of neoantigens with monoclonal antibodies. *J Immunol* **129**, 445-447 (1982).

16. S. Uwatoko, S. Aotsuka, M. Okawa, Y. Egusa, R. Yokohari, C. Aizawa, K. Suzuki, Characterization of c1q-binding igg complexes in systemic lupus erythematosus. *Clin Immunol Immunopathol* **30**, 104-116 (1984).

17. J. S. Kleer, P. A. Rabatscher, J. Weiss, J. Leonardi, S. B. Vogt, A. Kieninger-Gräfitsch, C. Chizzolini, U. Huynh-Do, C. Ribi, M. Trendelenburg, Epitope-specific anti-c1q autoantibodies in systemic lupus erythematosus. *Front Immunol* **12**, 761395 (2021).

18. I. Tsacheva, M. Radanova, N. Todorova, T. Argirova, U. Kishore, Detection of autoantibodies against the globular domain of human c1q in the sera of systemic lupus erythematosus patients. *Mol Immunol* **44**, 2147-2151 (2007).

19. M. Radanova, V. Vasilev, B. Deliyska, U. Kishore, V. Ikonomov, D. Ivanova, Anti-c1q autoantibodies specific against the globular domain of the c1qb-chain from patient with lupus nephritis inhibit c1q binding to igg and crp. *Immunobiology* **217**, 684-691 (2012).

20. D. Vanhecke, L. T. Roumenina, H. Wan, M. Osthoff, M. Schaller, M. Trendelenburg, Identification of a major linear c1q epitope allows detection of systemic lupus erythematosus anti-c1q antibodies by a specific peptide-based enzyme-linked immunosorbent assay. *Arthritis Rheum* **64**, 3706-3714 (2012).

21. V. Giudicelli, X. Brochet, M. P. Lefranc, Imgt/v-quest: Imgt standardized analysis of the immunoglobulin (ig) and t cell receptor (tr) nucleotide sequences. *Cold Spring Harb Protoc* **2011**, 695-715 (2011).

22. C. A. Ogden, A. deCathelineau, P. R. Hoffmann, D. Bratton, B. Ghebrehiwet, V. A. Fadok, P. M. Henson, C1q and mannose binding lectin engagement of cell surface calreticulin and cd91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* **194**, 781-795 (2001).

23. R. Fong, K. Kajihara, M. Chen, I. Hotzel, S. Mariathasan, W. L. W. Hazenbos, P. J. Lupardus, Structural investigation of human s. Aureus-targeting antibodies that bind wall teichoic acid. *MAbs* **10**, 979-991 (2018).

24. S. A. Zwarthoff, K. Widmer, A. Kuipers, J. Strasser, M. Ruyken, P. C. Aerts, C. J. C. de Haas, D. Ugurlar, M. A. den Boer, G. Vidarsson, J. A. G. van Strijp, P. Gros, P. Parren, K. P. M. van Kessel, J. Preiner, F. J. Beurskens, J. Schuurman, D. Ricklin, S. H. M. Rooijakkers, C1q binding to surface-bound igg is stabilized by c1r(2)s(2) proteases. *Proc Natl Acad Sci U S A* **118** (2021).

25. Q. Y. Fang, F. Yu, Y. Tan, L. X. Xu, L. H. Wu, G. Liu, F. M. Shao, M. H. Zhao, Anti-c1q antibodies and igg subclass distribution in sera from chinese patients with lupus nephritis. *Nephrol Dial Transplant* **24**, 172-178 (2009).

26. I. E. Coremans, M. R. Daha, E. A. van der Voort, C. E. Siegert, F. C. Breedveld, Subclass distribution of iga and igg antibodies against clq in patients with rheumatic diseases. *Scand J Immunol* **41**, 391-397 (1995).

27. M. H. Van Regenmortel, D. Altschuh, J. Chatellier, L. Christensen, N. Rauffer-Bruyère, P. Richalet-Secordel, J. Witz, G. Zeder-Lutz, Measurement of antigen-antibody interactions with biosensors. *J Mol Recognit* **11**, 163-167 (1998).

28. J. J. Wisnieski, S. M. Jones, Comparison of autoantibodies to the collagen-like region of c1q in hypocomplementemic urticarial vasculitis syndrome and systemic lupus erythematosus. *J Immunol* **148**, 1396-1403 (1992).

29. T. H. Sharp, A. L. Boyle, C. A. Diebolder, A. Kros, A. J. Koster, P. Gros, Insights into igm-mediated complement activation based on in situ structures of igm-c1-c4b. *Proc Natl Acad Sci U S A* **116**, 11900-11905 (2019).

30. D. Ugurlar, S. C. Howes, B.-J. de Kreuk, R. I. Koning, R. N. de Jong, F. J. Beurskens, J. Schuurman, A. J. Koster, T. H. Sharp, P. W. Parren, Structures of c1-igg1 provide insights into how danger pattern recognition activates complement. *Science* **359**, 794-797 (2018).

31. P. F. Kerkman, E. Fabre, E. I. van der Voort, A. Zaldumbide, Y. Rombouts, T. Rispens, G. Wolbink, R. C. Hoeben, H. Spits, D. L. Baeten, T. W. Huizinga, R. E. Toes, H. U. Scherer, Identification and characterisation of citrullinated antigen-specific b cells in peripheral blood of patients with rheumatoid arthritis. *Ann Rheum Dis* **75**, 1170-1176 (2016).

32. M. L. Gonzalez, M. B. Frank, P. A. Ramsland, J. S. Hanas, F. J. Waxman, Structural analysis of igg2a monoclonal antibodies in relation to complement deposition and renal immune complex deposition. *Mol Immunol* **40**, 307-317 (2003).

33. G. Wang, R. N. de Jong, E. T. van den Bremer, F. J. Beurskens, A. F. Labrijn, D. Ugurlar, P. Gros, J. Schuurman, P. W. Parren, A. J. Heck, Molecular basis of assembly and activation of complement component c1 in complex with immunoglobulin g1 and antigen. *Mol Cell* **63**, 135-145 (2016).

34. C. S. M. Kramer, M. E. I. Franke-van Dijk, K. H. Bakker, M. Uyar-Mercankaya, G. E. Karahan, D. L. Roelen, F. H. J. Claas, S. Heidt, Generation and reactivity analysis of human recombinant monoclonal antibodies directed against epitopes on hla-dr. *Am J Transplant* **20**, 3341-3353 (2020).

35. R. H. Zubler, F. Erard, R. K. Lees, M. Van Laer, C. Mingari, L. Moretta, H. R. MacDonald, Mutant el-4 thymoma cells polyclonally activate murine and human b cells via direct cell interaction. *J Immunol* **134**, 3662-3668 (1985).

36. L. C. Lighaam, E. Vermeulen, T. Bleker, K. J. Meijlink, R. C. Aalberse, E. Barnes, E. L. Culver, S. M. van Ham, T. Rispens, Phenotypic differences between igg4+ and igg1+ b cells point to distinct regulation of the igg4 response. *J Allergy Clin Immunol* **133**, 267-270.e261-266 (2014).

37. S. Heidt, D. L. Roelen, C. Eijsink, M. Eikmans, C. van Kooten, F. H. Claas, A. Mulder, Calcineurin inhibitors affect b cell antibody responses indirectly by interfering with t cell help. *Clin Exp Immunol* **159**, 199-207 (2010).

38. C. S. M. Kramer, M. E. I. Franke-van Dijk, A. J. Priddey, T. Pongrácz, E. Gnudi, H. Car, G. E. Karahan, E. van Beelen, C. C. C. Zilvold-van den Oever, H. J. Rademaker, N. de Haan, M. Wuhrer, V. Kosmoliaptsis, P. Parren, A. Mulder, D. L. Roelen, F. H. J. Claas, S. Heidt, Recombinant human monoclonal hla antibodies of different igg subclasses recognising the same epitope: Excellent tools to study differential effects of donor-specific antibodies. *Hla* **94**, 415-424 (2019).

39. X. Brochet, M. P. Lefranc, V. Giudicelli, Imgt/v-quest: The highly customized and integrated system for ig and tr standardized v-j and v-d-j sequence analysis. *Nucleic Acids Res* **36**, W503-508 (2008).

40. M. Lo, H. S. Kim, R. K. Tong, T. W. Bainbridge, J. M. Vernes, Y. Zhang, Y. L. Lin, S. Chung, M. S. Dennis, Y. J. Zuchero, R. J. Watts, J. A. Couch, Y. G. Meng, J. K. Atwal, R. J. Brezski, C. Spiess, J. A. Ernst, Effector-attenuating substitutions that maintain antibody stability and reduce toxicity in mice. *J Biol Chem* **292**, 3900-3908 (2017).

41. A. F. Labrijn, J. I. Meesters, P. Priem, R. N. de Jong, E. T. van den Bremer, M. D. van Kampen, A. F. Gerritsen, J. Schuurman, P. W. Parren, Controlled fab-arm exchange for the generation of stable bispecific igg1. *Nat Protoc* **9**, 2450-2463 (2014).

42. C. A. Diebolder, F. J. Beurskens, R. N. de Jong, R. I. Koning, K. Strumane, M. A. Lindorfer, M. Voorhorst, D. Ugurlar, S. Rosati, A. J. Heck, J. G. van de Winkel, I. A. Wilson, A. J. Koster, R. P. Taylor, E. O. Saphire, D. R. Burton, J. Schuurman, P. Gros, P. W. Parren, Complement is activated by igg hexamers assembled at the cell surface. *Science* **343**, 1260-1263 (2014).

43. L. A. Trouw, S. C. Nilsson, I. Gonçalves, G. Landberg, A. M. Blom, C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. *Journal of Experimental Medicine* **201**, 1937-1948 (2005).

44. C. Moreau, I. Bally, A. Chouquet, B. Bottazzi, B. Ghebrehiwet, C. Gaboriaud, N. Thielens, Structural and functional characterization of a single-chain form of the recognition domain of complement protein c1q. *Front Immunol* **7**, 79 (2016).

45. J. R. Kremer, D. N. Mastronarde, J. R. McIntosh, Computer visualization of three-dimensional image data using imod. *J Struct Biol* **116**, 71-76 (1996).

46. E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, Ucsf chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**, 1605-1612 (2004).

47. T. Šuštić, J. Van Coillie, M. D. Larsen, N. I. L. Derksen, Z. Szittner, J. Nouta, W. Wang, T. Damelang, I. Rebergen, F. Linty, . . . G. Vidarsson, Immunoassay for quantification of antigen-specific igg fucosylation. *EBioMedicine* **81**, 104109 (2022).

48. E. Boero, I. Brinkman, T. Juliet, E. van Yperen, J. A. G. van Strijp, S. H. M. Rooijakkers, K. P. M. van Kessel, Use of flow cytometry to evaluate phagocytosis of staphylococcus aureus by human neutrophils. *Front Immunol* **12**, 635825 (2021).

49. A. R. Cruz, M. A. D. Boer, J. Strasser, S. A. Zwarthoff, F. J. Beurskens, C. J. C. de Haas, P. C. Aerts, G. Wang, R. N. de Jong, F. Bagnoli, J. A. G. van Strijp, K. P. M. van Kessel, J. Schuurman, J. Preiner, A. J. R. Heck, S. H. M. Rooijakkers, Staphylococcal protein a inhibits complement activation by interfering with igg hexamer formation. *Proc Natl Acad Sci U S A* **118** (2021).

Supporting information

Western blots of C1q in various conformational states

C1q in different conformational states was prepared in Laemmli sample buffer (Bio-Rad) as either unheated, heated for 5 minutes at 95°C, or heated and reduced by adding 5% 2-mercaptoethanol in the sample buffer. A total of 2 µg C1q was loaded per lane of a 4-15% gradient precast polyacrylamide gel (Bio-Rad) and run at 110 V for 1 hour. The PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher) served as reference for the molecular weights of the bands.

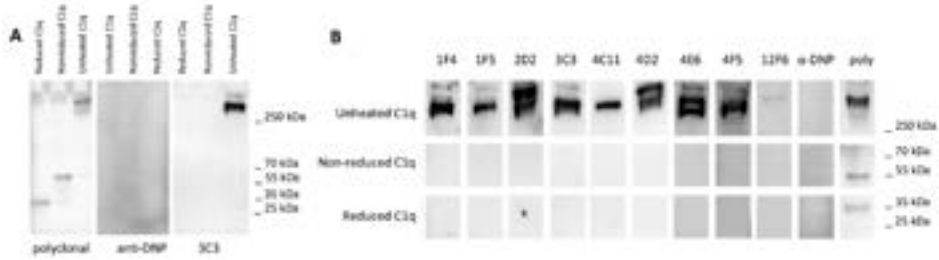
The gel was washed in ultrapure water and proteins were blotted to a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (both Bio-Rad). The blots were washed three times with ultrapure water and blocked with 30 mg/ml skim milk powder (Fluka) in PBS/0.05% Tween (blocking buffer) for 90 minutes. After washing two times with ultrapure water, the blots were cut into pieces to be stained with different mAbs and incubated with 5 µg/ml anti-C1q mAb or 1:20,000 rabbit anti-C1q polyclonal antibody in blocking buffer for 1 hour. The blots were washed three times with PBS/0.05% Tween, then incubated with 1:1000 rabbit anti-human IgG-HRP (DAKO), or with 1:20,000 goat anti-rabbit-HRP (DAKO) matching the primary antibodies. After 1 hour, blots were washed three times with PBS/0.05% Tween and once with ultrapure water. Blots were then incubated in ECL blotting reagent (Cytiva) and imaged on ChemiDoc MP Imaging System (Bio-Rad).

ELISA competition between anti-C1q Fab2 and biotinylated SLE antibodies

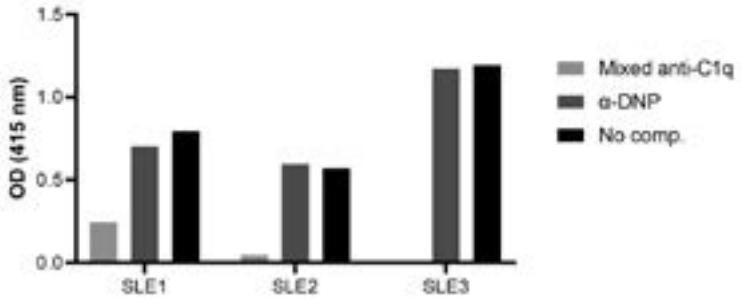
Plates and buffers from the QUANTA Lite Anti-C1q ELISA (Werfen) were used in this assay. Fab2 fragments of anti-C1q mAbs were made using FabRICATOR enzyme (Genovis) and protein A-purified antibodies from SLE patients and anti-C1q negative donors were biotinylated with the Pierce Antibody Biotinylation Kit (ThermoFisher). Mixed Fab2 of anti-C1q mAbs 1F4, 1F5, 4D2 and 4F5 were diluted in Sample Diluent to 50 µg/ml IgG equivalent final concentration (per mAb) and incubated on the plate for 1 hour at room temperature. Without washing, 25 µg/ml biotinylated purified serum antibodies in Sample Diluent were added and incubated 1 hour at room temperature. Plates were washed in HRP wash buffer and 0.2 µg/ml streptavidin-HRP was incubated for 1 hour at room temperature. After washing with HRP Wash buffer, ABTS/0.015% H_2O_2 was added for detection. Absorbance was measured at 415 nm using a microplate reader. Average absorbance for anti-C1q negative serum samples was subtracted from absorbance for SLE samples.

ELISA screening for anti-C1q binding to linear peptides

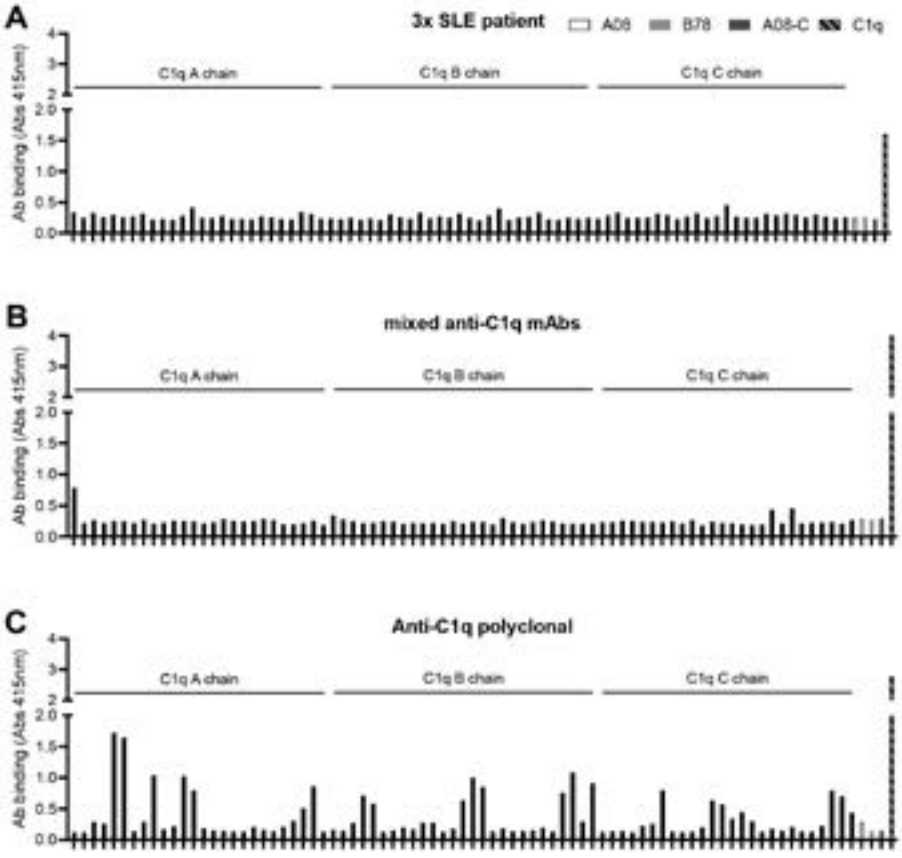
A comprehensive set of peptides covering the A, B and C chains of C1q was produced at the in-house peptide facility of the LUMC. Peptides were 21 amino acids long, overlapped 12 amino acids with the next peptide, and were C-terminally biotinylated after an aminohexanoic acid linker. Additionally, all cysteine residues were converted to α-aminobutyric acid (Abu), which better resembles a cysteine in disulphide bond as it would be in the full C1q protein. Previously described peptides A08, B78, and negative control A08-C, biotinylated as described above, were also tested [20]. Peptides were initially dissolved in DMSO, then diluted 1000x in PTB to 10 µg/ml and incubated on streptavidin coated plates (ThermoFisher) for 1 hour at 37°C. After washing, plates were incubated with a mix of the anti-C1q mAbs (5 µg/ml for each mAb), a mix of anti-C1q positive plasma samples (each 1:50 diluted), or 1:1000 rabbit anti-C1q polyclonal in PTB buffer for 1 hour at 37°C. After washing, bound antibodies were detected with matched rabbit anti-human IgG-HRP or goat anti-rabbit-HRP for the polyclonal and plates were developed by incubating with ABTS/0.015% H_2O_2 and measured using a microplate reader.



Supplementary Figure 1. Western blot of C1q in various states of protein folding, with detection by anti-C1q mAbs. (A) Full blots, as example, with C1q detection by rabbit polyclonal anti-C1q, anti-DNP mAb, and anti-C1q mAb 3C3, on unheated C1q, heated but nonreduced C1q and reduced C1q. (B) Blots were made for all anti-C1q mAbs, and relevant parts were aligned as summary.



Supplementary Figure 2. Competition between biotinylated SLE antibodies and mixed anti-C1q. Binding to C1q was tested for biotinylated SLE antibodies without competition, with a negative control antibody, or in the presence of mixed Fab2 fragments of mAbs 1F4, 1F5, 4D2 and 4F5.



Supplementary Figure 3. Binding of anti-C1q antibodies to linear C1q peptides. Binding to biotinylated linear C1q peptides of the A, B and C chains, and also previously described A08 and B78 peptides was screened in ELISA. Antibody binding was tested for (A) a mix of three anti-C1q positive SLE patient plasma samples, (B) a mix of all nine anti-C1q mAbs and (C) a polyclonal rabbit anti-C1q antibody as positive control.

CHAPTER 7

General discussion

Douwe J. Dijkstra

Complement and complement therapeutics in rheumatic disease

The human complement system plays a major role in immune defense and maintenance of homeostasis, but through overactivation, inadequate regulation or improper function, complement is also involved in disease processes. This detrimental role of the complement system is apparent for example in several rheumatic diseases, as we have reviewed in **chapter 2**. In rheumatoid arthritis (RA), the observation that activated complement protein fragments are present in the synovium and in the circulation was an initial clue for a role for complement activation in the disease processes of RA [1-7]. This was experimentally elaborated in mouse models of collagen antibody-induced arthritis (CAIA), where especially the alternative complement pathway was essential for disease development, with only a minor role for the classical pathway [8-10]. Meanwhile, the influence of complement on systemic lupus erythematosus (SLE) can take on of two forms. Complement deposition in affected organs and decreased concentrations of circulating complement proteins due to massive consumption of complement is often observed in SLE patients and correlates with disease severity, implicating that complement activation contributes to the disease processes of SLE [11-13]. On the other hand, individuals who are genetically deficient for C1q (and to a lesser extent also C1r, C1s, C4 and C2) are highly likely to develop SLE disease [14, 15]. According to the waste disposal hypothesis, the inability of the classical complement pathway to help clear apoptotic cells and debris leads to the exposure of intracellular antigens and formation of autoantibodies against these antigens. Additionally, C1q is reported to have a regulatory effect on T cells, interacting with $CD8^+$ T cells with their globular head to modulate T-cell metabolism [16]. The absence of this pathway may increase the occurrence of autoimmunity.

Even though a large body of evidence points towards the involvement of the complement system in both RA and SLE, therapeutics targeting complement have so far not yielded convincing results. Eculizumab, an antibody that prevents the cleaving and activation of C5, was clinically tested in both RA and SLE patients, but did not progress to pivotal clinical trials and regulatory approval [17]. Off-label use of eculizumab anecdotally demonstrated positive outcomes in lupus nephritis, as reported by in several single cases [18, 19], but no large-scale study has been performed. These results show that complement activation, at least when inhibited at the level of C5, is not likely to play an essential role in the immunopathology of established RA and SLE, and C5 is therefore not the best target for therapy of these diseases.

Successful intervention by complement-targeting therapeutics is possible in other diseases, exemplified by the approval of anti-C5 antibodies eculizumab and ravulizumab in paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) [20]. In **chapter 2**, the C5a receptor-blocking small molecule avacopan was described to be in clinical trials, and it has since been approved for the treatment of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) [21-23]. In AAV, neutrophils are attracted and activated through their C5a receptor and subsequently cause more complement activation by secreted factors and neutrophil extracellular traps (NET). This feedback loop is broken by blockade of the C5a receptor, as has now been shown in human studies with avacopan [24, 25].

With these and other complement-targeting therapeutics on the market, and several more under clinical investigation, every trial allows the scientific community to learn more about the relative contribution of complement in the respective disease [20]. In this sense, it will be interesting to follow a recently developed antibody against C1q, which aims to deplete free C1q from the circulation [26]. As C1q deficiency is associated with development of SLE disease, long-term treatment with this anti-C1q antibody may have as a side-effect the development of autoimmune disease. The currently approved complement-targeting therapeutics work systemically, which may have a great impact on side effects. Even though safety concerns for eculizumab are less prominent than once feared, inhibiting (parts of) complement systemically to treat a localized disease remains sub-optimal [27]. This leaves a great opportunity for future therapeutics in targeting specific locations within the body. Localizing the therapeutic to the affected organ or tissue will allow the next generation of complement drugs to maintain normal complement functionality throughout the rest of the body [van de Bovenkamp *et al.*, submitted].

C1q as biomarker in tuberculosis disease

While complement is a therapeutic target in some diseases, complement components may serve as a biomarker for other diseases. Tuberculosis (TB) is a clear example of such a disease where current diagnostic approaches are either very time-consuming in the case of bacterial culture from sputum, or incapable of distinguishing between active disease, latent infection or past infection in the case of immunological tests. This raises the need for better TB diagnostics, and complement protein C1q has been identified as a possible biomarker. We and others have found that the C1q concentration in serum is increased in patients with active pulmonary TB disease compared to latently infected patients and relevant disease controls [28-30]. Whole blood transcriptomics analyses also

revealed other possible biomarkers, including differential expression of certain interferon-stimulated genes. One particular study selected 10 of these genes to form a whole blood transcriptional type-1 interferon signature for use as a diagnostic marker [31]. In **chapter 3**, we observed an inverse correlation between C1q and the interferon signature in active pulmonary TB patients. As a result, all patients in the cohort were positive for high C1q concentration or for high type-1 interferon signature, with some double positive patients. Importantly, none of the active TB patients were double negative, while a large majority of healthy controls fell within the double negative quadrant. We conclude that the use of both C1q and the interferon signature yields improved diagnostic value based on the results in our cohort, compared to either of the single biomarkers.

TB can spread to many different organs, and we also investigated involvement of the eye, in the form of TB uveitis, which is especially difficult to diagnose as it may present itself clinically similarly to other ocular conditions. Additionally, association between uveitis and positive immunological tests for TB may be coincidental in regions with high rates of latent TB infection. In uveitis of unknown cause, we could stratify patients into groups based on low or high risk of TB uveitis by using both C1q and the interferon signature as combined biomarkers. Although further development and validation are still needed, the inverse correlation between C1q concentration and the type-1 interferon signature we observed for active pulmonary TB in **chapter 3** means they could work well together. The main advantage over the current immunological tests is their ability to distinguish active disease from past exposure. Bacterial culture from a biological sample can also achieve this distinction, but may take weeks to deliver results. The use of C1q concentration and interferon signature therefore offers the opportunity to start anti-tuberculosis treatment rapidly, without the risk of overzealous treatment in patients with past exposure to TB. We investigated TB in the context of the lungs and eyes, however it is likely that a similar strategy would work for TB involvement with other organs.

To persist in the body, *Mycobacterium tuberculosis*, the causative agent of TB infection, needs to evade the immune system. At a later time however, the bacterium also causes inflammation to aid transmission [32]. It is likely that the increased C1q concentration during active TB disease is a result of immunomodulation by the bacterium. According to our current hypothesis, it could be beneficial for *M. tuberculosis* to stimulate C1q production, which is known to have a regulatory influence on T cells [16]. Actual complement activation may not be increased, since an increase in systemic levels of the regulator C1-INH was also observed [33]. Higher C1-INH levels can counteract any increased complement cascade initiation that could otherwise be caused by higher C1q

levels. It is currently unknown exactly how *M. tuberculosis* would instruct the human body to produce more C1q.However, we do know that the additional C1q production occurs systemically, as both increased C1q protein and increased C1q gene expression are detected in whole blood. We recently identified that among the circulating cells a specific subsets of monocytes are the main producers of C1q during active TB disease [34]. Further research will have to elucidate through which signals and pathways *M. tuberculosis* or TB-infected macrophages in the lung granuloma instruct upregulation of C1q in circulating cells.

Increased C1q concentration can serve as a biomarker for active TB disease, and may be used in clinical diagnostics to identify active TB disease, possibly in combination with other biomarkers. In light of spreading (multi)drug-resistant TB strains, therapy to stop immune evasion by *M. tuberculosis* and allow the immune system itself to clear the bacterium, should be investigated [35]. As increased C1q may play an immunomodulatory role during active TB disease, it could be a target in those cases where traditional drugs fail to eradicate the infection. Systemically lowering the C1q concentration over a prolonged period of time may be difficult and may have side-effects. However, blocking the downstream effects of high C1q concentrations may provide an opportunity. The initial literature describing interaction between C1q and T cells in autoimmunity hypothesized that C1q can alter $CD8^+$ T-cell function through metabolism [16]. According to their theory, this would involve the receptor for the globular heads of C1q (gC1qR), which is expressed primarily on mitochondria, but also on the cell surface [36]. When the interactions between C1q and T cells are further elucidated, an inhibitor of this interaction or its downstream signaling could be interesting in unleashing T cells to fight TB infection, analogous to the immune checkpoint inhibition which has revolutionized cancer treatment in the last decade [37].

The role of complement in pregnancy and preeclampsia

We investigated C1q and also Factor H in a very different situation in **chapter 4**: pregnancy and the pregnancy complication preeclampsia. During pregnancy, shaping and maintaining the placenta requires a large amount of blood vessel formation, tissue remodeling and subsequently the resulting cellular debris has to be cleared. These are processes in which C1q is known to play a role throughout the body. It is therefore interesting that we observed higher serum C1q concentrations in pregnant women than in nonpregnant controls. Similarly, we also observed higher serum factor H concentration in pregnant women compared to nonpregnant controls, as has been reported before [38, 39]. We postulate that C1q is upregulated during pregnancy to

perform its role in neovascularization and removal of debris from tissue remodeling and invasion of fetal trophoblasts, while increased factor H helps limit alternative pathway complement activation which is known to occur on apoptotic and necrotic bodies [40].

Preeclampsia is an important complication of pregnancy, and a large contributor to maternal and fetal morbidity. It presents during the second half of pregnancy with increased blood pressure and typically also proteinuria. While the etiology is not clearly understood, this condition is thought to originate in improper placentation, a partly dysfunctional placenta, and the stress signals this causes when the fetus grows larger and needs more nutrition [41]. In our research in **chapter 4** we showed that serum factor H concentration was lower in preeclampsia patients than in pregnant women in a control group without preeclampsia. This difference was particularly found in early-onset preeclampsia patients (defined as gestational age below 34 weeks at delivery), which are generally the more severe cases. These findings are in agreement with earlier reports of increased concentrations of the alternative pathway activation fragment Bb in preeclampsia cases [39, 42, 43].

The only known cure for preeclampsia is delivery of the fetus and placenta, although preventative administration of aspirin during high-risk pregnancies has been used with positive results [44]. A better understanding of preeclampsia is therefore needed to improve treatment [45]. The study presented in **chapter 4** indicates a role for (the lower concentration of) factor H in preeclampsia, however it has not been established whether it would be involved in the cause of preeclampsia, or rather be a step in the later pathology. Alternatively, the lower factor H levels may also be a result of increased binding to tissues in the prevention of further pathology. On account of the relatively small differences in factor H concentration between preeclampsia and control pregnancies, and the fact that our study was performed on samples from the end of pregnancy, factor H would not be an obvious biomarker candidate based on this study. A follow-up of this study should therefore focus on a further contribution to the understanding of preeclampsia. To make more definitive statements on a role for factor H in the cause of preeclampsia, factor H would have to be monitored prospectively, before the onset of preeclampsia, in a large cohort of pregnancies. Such a study should aim to learn at which point during the pathogenesis of preeclampsia the decreased concentration of factor H develops, and subsequently whether a therapeutic strategy involving factor H would be feasible.

Autoantibodies to solid-phase C1q

Autoantibodies against C1q are investigated in **chapters 4, 5 and 6** of this thesis. These autoantibodies have been known for decades and are associated with autoimmune disease, mostly with lupus nephritis [46-48]. Interestingly however, anti-C1q autoantibodies are also present in a few percent of the general population, without current signs, or evidence for future development, of autoimmune disease [49]. Interestingly, these healthy individuals with anti-C1q autoantibodies often have normal circulating levels of C1q. This indicates that the anti-C1q antiantibodies apparently do not make complexes with C1q that would lead to their clearance. This is likely explained by the peculiar characteristic of these autoantibodies that they specifically bind to solid-phase C1q, which means C1q that is bound to a ligand or surface. In this situation, C1q undergoes a conformational change, opening up a cryptic epitope which anti-C1q autoantibodies bind to. As these autoantibodies bind to C1q specifically when C1q is engaged with a ligand, they may cause unwanted amplification of immune responses in this location.

Due to the association with autoimmune disease and the hypothesis that anti-C1q binding could contribute further immune activation in a local environment, anti-C1q autoantibodies have been investigated as a biomarker in many diseases. An association between anti-C1q and a certain disease means testing for anti-C1q could aid diagnosis. One such report identified anti-C1q as a possible biomarker for lung involvement in systemic sclerosis [50]. Systemic sclerosis is characterized by fibrosis of the skin and internal organs, with most related deaths attributed to involvement of the lungs and heart. In **chapter 5**, we examined the relation between anti-C1q autoantibodies, systemic sclerosis and its associated lung conditions, most commonly pulmonary fibrosis and pulmonary arterial hypertension. Due to the severity of these conditions, it would be of considerable importance to have a prognostic marker for them as several treatment options are available [51]. However, our study did not support a prognostic value of anti-C1q autoantibodies in systemic sclerosis or its related lung conditions. The systemic sclerosis patients positive for anti-C1q did show a much higher incidence of anti-topoisomerase antibodies, which are themselves reported to associate with lung complications and more severe disease [52]. The overlap between anti-C1q and anti-topoisomerase antibodies would detract from any prognostic value of anti-C1q, therefore anti-C1q autoantibodies are unlikely to be an adequate biomarker for systemic sclerosis or its related lung conditions.

Anti-C1q autoantibodies have been studied in the context of pregnancy as well, multiple studies previously linked anti-C1q to different negative pregnancy outcomes,

but not to preeclampsia [53-56]. In **chapter 4**, we also studied anti-C1q in pregnancy and in preeclampsia patients, and found no significant association between anti-C1q and preeclampsia in the three pooled cohorts from the Netherlands, Finland and Norway. A recent study from Italy concluded that anti-C1q autoantibody levels were actually lower in preeclampsia than in control pregnancy, which we also observed when looking only at the cohort from the Netherlands [57]. However, our other two cohorts did contain equal or higher percentages of anti-C1q positive individuals among preeclampsia patients compared to controls. Careful investigation of demographic and clinical data available did not explain these differences in the population from Italy and the Netherlands versus Finland and Norway. Overall, the two studies combined do not indicate a role for anti-C1q in the pathogenesis of preeclampsia.

Characterization of anti-C1q autoantibodies

Anti-C1q autoantibodies have been studied for decades, largely using serum as a source. This approach has multiple drawbacks, such as the forced focus on polyclonal mix of antibodies and the high salt concentration required to distinguish anti-C1q from immune complexes. To overcome these drawbacks, we set out to isolate C1q-reactive B cells and recombinantly reproduce human anti-C1q autoantibodies in **chapter 6**. We were indeed able to successfully obtain 9 unique clones from 4 different donors, allowing their characterization. We showed that at least two unique epitopes on the C1q protein are targeted by anti-C1q autoantibodies, showcasing their diversity, and that these antibodies indeed specifically bind solid-phase C1q as was described in literature for polyclonal autoantibodies [47, 58, 59]. Since we studied a relatively limited selection of antibodies, it is entirely possible that even more unique epitopes for anti-C1q exist. Furthermore, electron microscopy revealed that multiple molecules of the same anti-C1q monoclonal antibody could bind to the same C1q protein. Although this is not unexpected due to the radial symmetry of C1q, no prior study had found evidence that multiple antibodies would bind to one C1q molecule. All 9 anti-C1q antibodies characterized in this study originated from healthy donors. In ELISA competition assays, we observed that the anti-C1q monoclonals and antibodies purified from SLE patients compete with each other for binding to C1q, showing they target the same or similar (overlapping) epitopes on C1q. Additionally, we showed that the anti-C1q monoclonal antibodies are specific for solid-phase C1q and they were of the IgG isotype, which is also the main isotype of anti-C1q autoantibodies in SLE patients [60, 61]. Based on these factors, we argue that the results in this study are very likely to translate to the anti-C1q autoantibodies found in SLE patients.

The notion that anti-C1q autoantibodies in healthy subjects are identical to those in SLE patients, where they especially associate with lupus nephritis, raises the question why these autoantibodies are harmful in one person but not in the other. It is conceivable that for local pathology to occur, the target of these autoantibodies must be abundantly present and this organ or location must be vulnerable to the effector mechanisms mediated by the autoantibodies. In the case of lupus nephritis, this location is the kidney, more specifically the glomerulus. Indeed, in a mouse model of lupus nephritis, renal pathology by anti-C1q is only observed in the presence of C1q-containing immune complexes [62]. This same study also found that renal damage depended on C1q, complement activation and also on Fcγ receptors. In **chapter 6**, we found that addition of anti-C1q to C1q-containing immune complexes did not increase complement activation. We also investigated Fcγ receptors and observed that their binding to immune complexes markedly decreased by addition of C1q, probably because C1q occupies or masks the binding site for Fcγ receptors on IgG as reported recently [63]. Reversely, binding of Fcγ receptors increased again after subsequent addition of anti-C1q antibodies. We unite these results in **chapter 6** and earlier results by Trouw *et al.*, to hypothesize that complement activation by immune complexes deposited in the glomeruli attracts immune cells, while anti-C1q antibodies on the C1q-containing immune complexes engages Fcγ receptors on these cells to cause the actual renal pathology of lupus nephritis (Figure 1). This model is further supported by a recent finding that administration of C5a receptor-blocking small molecule avacopan in a mouse model of lupus nephritis severely reduced influx of granulocytes and prevented kidney damage [64].

In order for anti-C1q antibodies to be present in the circulation, whether in a healthy individual or in an SLE patient, C1q-reactive B cells must arise. Immature B cells that recognize self-antigens with high affinity in the bone marrow or spleen should undergo apoptosis [65]. In the case of anti-C1q it is less likely that immature B cells will encounter solid-phase C1q however, which may allow them to escape and mature, whereas any B cells recognizing fluid-phase C1q would fall victim of negative selection. Mature B cells may be stimulated upon encountering their antigen in a T cell-dependent or independent manner. T cell-independent stimulation usually occurs for non-protein antigens, based on multivalency to crosslink the B-cell receptor and C3d deposition to stimulate complement receptor 2 [66]. Interestingly, C1q is also multivalent, as we showed that multiple antibodies can bind at once, and solid-phase C1q is in an activated state and will therefore be in close proximity of deposited C3. Still, based on the characteristics of anti-C1q autoantibodies, it is likely they are the product of

T-cell stimulated B cells. The autoantibodies we isolated in **chapter 6** were all of the IgG isotype and many contained a reasonable amount of V-gene mutations. These features are typical of germinal center reactions, which require T-cell help based on peptides presented in class II major histocompatibility complex (MHC) on the B cell. It is unlikely that these helper T cells specifically recognize a peptide of C1q, as they would have been selected out, but they likely recognize a foreign protein to which C1q has bound. A B cell producing anti-C1q would thereby receive help from a T cell recognizing an unrelated foreign protein, analogous to the hapten-carrier effect, but now with two proteins in complex. This is a possible way how anti-C1q-producing B cells can arise and continue to exist and produce these potentially harmful autoantibodies.

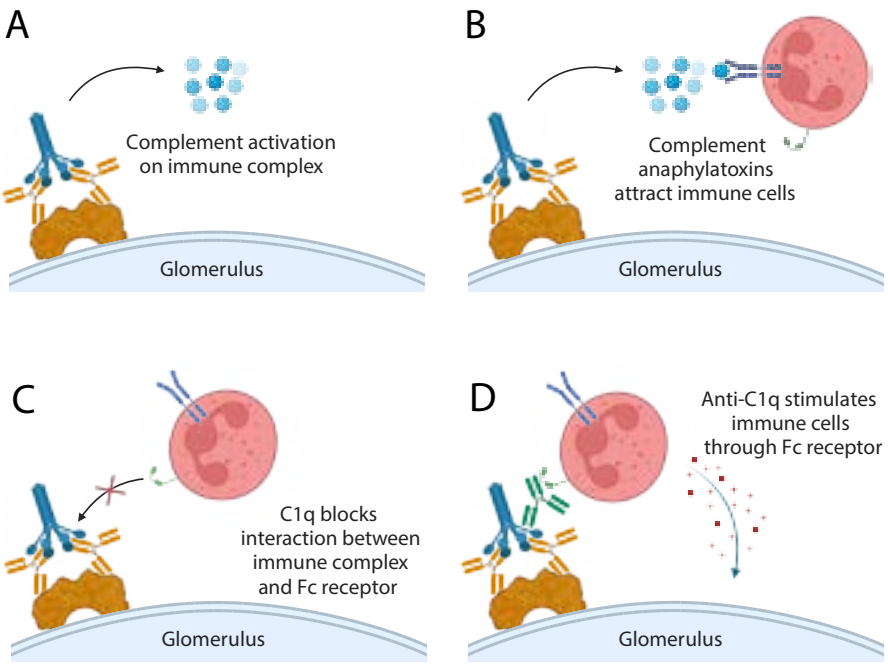


Figure 1. The proposed influence of complement activation, Fc receptors and anti-C1q autoantibodies in the pathology of lupus nephritis. (A) Deposited immune complexes in the glomerulus activate the complement system. (B) Several products of complement activation serve as anaphylatoxins, which are able to attract immune cells such as granulocytes to the glomerulus. (C) The Fc receptors on the newly attracted immune cells are blocked from interacting with immune complexes by the presence of C1q, which masks the binding site for Fc receptors on antibodies. (D) The binding of anti-C1q autoantibodies to C1q-containing immune complexes offers a target for Fc receptors, which causes the immune cells to activate. This process then leads to glomerular inflammation and renal damage in lupus nephritis patients. Created with BioRender.

While anti-C1q autoantibodies can contribute to autoimmune disease, their isolation and reproduction, as described in **chapter 6**, opens the door to new opportunities as well. By specifically recognizing solid-phase C1q, these antibodies are essentially directed towards location where classical pathway complement activation occurs. When coupled to a tracer, these antibodies or their derived Fab fragments may be used to trace C1q binding in tissues, for instance in patients with suspected autoimmune disease or transplant rejection, without the need to take a biopsy. Engineered anti-C1q may also be employed in a therapeutic setting. An aspect of the anti-C1q antibody research we did not describe in this thesis, involves bispecific antibodies targeting both solid-phase C1q and a complement inhibitor such as factor H or C4b binding protein. These engineered antibodies are able to (partially) inhibit complement-mediated cell death in vitro. While further research is required to show efficacy and specificity in more complex models, the engineered anti-C1q antibodies do have potential to concentrate autologous complement inhibitors at the site of C1q activation. Thereby, the antibodies isolated in **chapter 6** may be developed into useful therapeutics for the treatment of disease where classical pathway complement activation plays a deleterious role, such as lupus nephritis.

The research described in this thesis underlines the many roles the complement system, and particularly C1q, play in human physiology and disease. Our research underlines the potential diagnostic value of C1q concentration in active TB disease and ocular involvement in TB. Next to C1q's role as a biomarker, it may also be a target for therapy in TB patients with high C1q levels. If C1q is indeed vital in the regulation of T-cell activity in TB, as proposed here, blocking C1q or its downstream signaling offers great possibilities for therapy to unlock the immune system. C1q also is the target of relatively common human autoantibodies. By studying these autoantibodies at a monoclonal instead of polyclonal level, we gained new insights into both their molecular characteristics and opened the door to new therapeutic opportunities. Most of all, this research urges continuation and validation to increase our understanding of canonical and non-canonical complement biology and hopefully contribute to future therapies.

References

1. J. P. Brodeur, S. Ruddy, L. B. Schwartz, G. Moxley, Synovial fluid levels of complement sc5b-9 and fragment bb are elevated in patients with rheumatoid arthritis. *Arthritis Rheum* **34**, 1531-1537 (1991).
2. A. J. Swaak, A. Van Rooyen, O. Planten, H. Han, O. Hattink, E. Hack, An analysis of the levels of complement components in the synovial fluid in rheumatic diseases. *Clin Rheumatol* **6**, 350-357 (1987).
3. G. Moxley, S. Ruddy, Elevated c3 anaphylatoxin levels in synovial fluids from patients with rheumatoid arthritis. *Arthritis Rheum* **28**, 1089-1095 (1985).
4. T. E. Mollnes, A. Paus, Complement activation in synovial fluid and tissue from patients with juvenile rheumatoid arthritis. *Arthritis Rheum* **29**, 1359-1364 (1986).
5. R. D. Inman, P. C. Harpel, C1 inactivator-c1s complexes in inflammatory joint disease. *Clin Exp Immunol* **53**, 521-528 (1983).
6. M. Shingu, Y. Watanabe, K. Tomooka, K. Yoshioka, E. Ohtsuka, M. Nobunaga, Complement degradation products in rheumatoid arthritis synovial fluid. *Br J Rheumatol* **33**, 299-300 (1994).
7. D. Wouters, A. E. Voskuyl, E. T. Molenaar, B. A. Dijkmans, C. E. Hack, Evaluation of classical complement pathway activation in rheumatoid arthritis: Measurement of c1q-c4 complexes as novel activation products. *Arthritis Rheum* **54**, 1143-1150 (2006).
8. N. K. Banda, K. Takahashi, A. K. Wood, V. M. Holers, W. P. Arend, Pathogenic complement activation in collagen antibody-induced arthritis in mice requires amplification by the alternative pathway. *J Immunol* **179**, 4101-4109 (2007).
9. N. K. Banda, J. M. Thurman, D. Kraus, A. Wood, M. C. Carroll, W. P. Arend, V. M. Holers, Alternative complement pathway activation is essential for inflammation and joint destruction in the passive transfer model of collagen-induced arthritis. *J Immunol* **177**, 1904-1912 (2006).
10. H. Ji, K. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, A. Ezekowitz, M. C. Carroll, M. Brenner, R. Weissleder, J. S. Verbeek, V. Duchatelle, C. Degott, C. Benoist, D. Mathis, Arthritis critically dependent on innate immune system players. *Immunity* **16**, 157-168 (2002).
11. A. J. Swaak, J. Groenwold, W. Bronsveld, Predictive value of complement profiles and anti-dsdna in systemic lupus erythematosus. *Ann Rheum Dis* **45**, 359-366 (1986).
12. G. Senaldi, V. A. Makinde, D. Vergani, D. A. Isenberg, Correlation of the activation of the fourth component of complement (c4) with disease activity in systemic lupus erythematosus. *Ann Rheum Dis* **47**, 913-917 (1988).
13. S. Manzi, J. E. Rairie, A. B. Carpenter, R. H. Kelly, S. P. Jagarlapudi, S. M. Sereika, T. A. Medsger, Jr., R. Ramsey-Goldman, Sensitivity and specificity of plasma and urine complement split products as indicators of lupus disease activity. *Arthritis Rheum* **39**, 1178-1188 (1996).
14. J. Leffler, A. A. Bengtsson, A. M. Blom, The complement system in systemic lupus erythematosus: An update. *Ann Rheum Dis* **73**, 1601-1606 (2014).
15. G. Sturfelt, L. Truedsson, Complement in the immunopathogenesis of rheumatic disease. *Nat Rev Rheumatol* **8**, 458-468 (2012).

16. G. S. Ling, G. Crawford, N. Buang, I. Bartok, K. Tian, N. M. Thielens, I. Bally, J. A. Harker, P. G. Ashton-Rickardt, S. Rutschmann, J. Strid, M. Botto, C1q restrains autoimmunity and viral infection by regulating cd8(+) t cell metabolism. *Science* **360**, 558-563 (2018).

17. M. L. Barilla-Labarca, K. Toder, R. Furie, Targeting the complement system in systemic lupus erythematosus and other diseases. *Clin Immunol* **148**, 313-321 (2013).

18. R. Coppo, L. Peruzzi, A. Amore, S. Martino, L. Vergano, I. Lastauka, A. Schieppati, M. Noris, P. A. Tovo, G. Remuzzi, Dramatic effects of eculizumab in a child with diffuse proliferative lupus nephritis resistant to conventional therapy. *Pediatr Nephrol* **30**, 167-172 (2015).

19. M. C. Pickering, M. Ismajli, M. B. Condon, N. McKenna, A. E. Hall, L. Lightstone, H. Terence Cook, T. D. Cairns, Eculizumab as rescue therapy in severe resistant lupus nephritis. *Rheumatology (Oxford)* **54**, 2286-2288 (2015).

20. E. E. West, T. Woodruff, V. Fremeaux-Bacchi, C. Kemper, Complement in human disease: Approved and up-and-coming therapeutics. *Lancet* 10.1016/s0140-6736(23)01524-6 (2023).

21. D. R. W. Jayne, A. N. Bruchfeld, L. Harper, M. Schaier, M. C. Venning, P. Hamilton, V. Burst, F. Grundmann, M. Jadoul, I. Szombati, V. Tesar, M. Segelmark, A. Potarca, T. J. Schall, P. Bekker, Randomized trial of c5a receptor inhibitor avacopan in anca-associated vasculitis. *J Am Soc Nephrol* **28**, 2756-2767 (2017).

22. P. A. Merkel, J. Niles, R. Jimenez, R. F. Spiera, B. H. Rovin, A. Bomback, C. Pagnoux, A. Potarca, T. J. Schall, P. Bekker, Adjunctive treatment with avacopan, an oral c5a receptor inhibitor, in patients with antineutrophil cytoplasmic antibody-associated vasculitis. *ACR Open Rheumatol* **2**, 662-671 (2020).

23. D. R. W. Jayne, P. A. Merkel, T. J. Schall, P. Bekker, Avacopan for the treatment of anca-associated vasculitis. *N Engl J Med* **384**, 599-609 (2021).

24. H. Xiao, A. Schreiber, P. Heeringa, R. J. Falk, J. C. Jennette, Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol* **170**, 52-64 (2007).

25. D. Huugen, A. van Esch, H. Xiao, C. J. Peutz-Kootstra, W. A. Buurman, J. W. Tervaert, J. C. Jennette, P. Heeringa, Inhibition of complement factor c5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int* **71**, 646-654 (2007).

26. J. A. Lansita, K. M. Mease, H. Qiu, T. Yednock, S. Sankaranarayanan, S. Kramer, Nonclinical development of anx005: A humanized anti-c1q antibody for treatment of autoimmune and neurodegenerative diseases. *Int J Toxicol* **36**, 449-462 (2017).

27. P. Hillmen, P. Muus, A. Röth, M. O. Elebute, A. M. Risitano, H. Schrezenmeier, J. Szer, P. Browne, J. P. Maciejewski, J. Schubert, A. Urbano-Ispizua, C. de Castro, G. Socié, R. A. Brodsky, Long-term safety and efficacy of sustained eculizumab treatment in patients with paroxysmal nocturnal haemoglobinuria. *Br J Haematol* **162**, 62-73 (2013).

28. R. Lubbers, J. S. Sutherland, D. Goletti, R. A. de Paus, C. H. M. van Moorsel, M. Veltkamp, S. M. T. Vestjens, W. J. W. Bos, L. Petrone, F. Del Nonno, . . . L. A. Trouw, Complement component c1q as serum biomarker to detect active tuberculosis. *Front Immunol* **9**, 2427 (2018).

29. Y. Cai, Q. Yang, Y. Tang, M. Zhang, H. Liu, G. Zhang, Q. Deng, J. Huang, Z. Gao, B. Zhou, C. G. Feng, X. Chen, Increased complement c1q level marks active disease in human tuberculosis. *PLoS One* **9**, e92340 (2014).

30. K. Dijkman, R. Lubbers, N. V. Borggreven, T. H. M. Ottenhoff, S. A. Joosten, L. A. Trouw, F. A. W. Verreck, Systemic and pulmonary c1q as biomarker of progressive disease in experimental non-human primate tuberculosis. *Sci Rep* **10**, 6290 (2020).

31. R. La Distia Nora, R. Sitompul, M. Bakker, M. A. Versnel, S. M. A. Swagemakers, P. J. van der Spek, M. Susiyanti, L. Edwar, S. Sjamsoe, G. Singh, R. D. Handayani, A. Rothova, P. M. van Hagen, W. A. Dik, Type 1 interferon-inducible gene expression in quantiferon gold tb-positive uveitis: A tool to stratify a high versus low risk of active tuberculosis? *PLoS One* **13**, e0206073 (2018).

32. P. Chandra, S. J. Grigsby, J. A. Philips, Immune evasion and provocation by mycobacterium tuberculosis. *Nat Rev Microbiol* **20**, 750-766 (2022).

33. R. Lubbers, J. S. Sutherland, D. Goletti, R. A. de Paus, D. J. Dijkstra, C. H. M. van Moorsel, M. Veltkamp, S. M. T. Vestjens, W. J. W. Bos, L. Petrone, S. T. Malherbe, G. Walzl, K. A. Gelderman, G. H. Groeneveld, A. Geluk, T. H. M. Ottenhoff, S. A. Joosten, L. A. Trouw, Expression and production of the serping1-encoded endogenous complement regulator c1-inhibitor in multiple cohorts of tuberculosis patients. *Mol Immunol* **120**, 187-195 (2020).

34. P. Niewold, D. J. Dijkstra, Y. Cai, D. Goletti, F. Palmieri, K. E. van Meijgaarden, F. A. W. Verreck, O. W. Akkerman, R. W. Hofland, E. M. Delemarre, S. Nierkens, M. K. Verheul, A. J. Pollard, J. T. van Dissel, T. H. M. Ottenhoff, L. A. Trouw, S. A. Joosten, Identification of circulating monocytes as producers of tuberculosis disease biomarker c1q. *Sci Rep* **13**, 11617 (2023).

35. N. Dookie, S. L. Ngema, R. Perumal, N. Naicker, N. Padayatchi, K. Naidoo, The changing paradigm of drug-resistant tuberculosis treatment: Successes, pitfalls, and future perspectives. *Clin Microbiol Rev* **35**, e0018019 (2022).

36. B. Ghebrehiwet, B. L. Lim, E. I. Peerschke, A. C. Willis, K. B. Reid, Isolation, cdna cloning, and overexpression of a 33-kd cell surface glycoprotein that binds to the globular “heads” of c1q. *J Exp Med* **179**, 1809-1821 (1994).

37. P. Sharma, S. Goswami, D. Raychaudhuri, B. A. Siddiqui, P. Singh, A. Nagarajan, J. Liu, S. K. Subudhi, C. Poon, K. L. Gant, S. M. Herbrich, S. Anandhan, S. Islam, M. Amit, G. Anandappa, J. P. Allison, Immune checkpoint therapy-current perspectives and future directions. *Cell* **186**, 1652-1669 (2023).

38. Z. Derzsy, Z. Prohászka, J. Rigó, Jr., G. Füst, A. Molvarec, Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol* **47**, 1500-1506 (2010).

39. K. Jia, L. Ma, S. Wu, W. Yang, Serum levels of complement factors c1q, bb, and h in normal pregnancy and severe pre-eclampsia. *Med Sci Monit* **25**, 7087-7093 (2019).

40. W. Xu, S. P. Berger, L. A. Trouw, H. C. de Boer, N. Schlagwein, C. Mutsaers, M. R. Daha, C. van Kooten, Properdin binds to late apoptotic and necrotic cells independently of c3b and regulates alternative pathway complement activation. *The Journal of Immunology* **180**, 7613-7621 (2008).

41. E. A. Steegers, P. von Dadelszen, J. J. Duvekot, R. Pijnenborg, Pre-eclampsia. *Lancet* **376**, 631-644 (2010).

42. M. C. Hoffman, K. K. Rumer, A. Kramer, A. M. Lynch, V. D. Winn, Maternal and fetal alternative complement pathway activation in early severe preeclampsia. *Am J Reprod Immunol* **71**, 55-60 (2014).

43. A. M. Lynch, J. R. Murphy, T. Byers, R. S. Gibbs, M. C. Neville, P. C. Giclas, J. E. Salmon, V. M. Holers, Alternative complement pathway activation fragment bb in early pregnancy as a predictor of preeclampsia. *Am J Obstet Gynecol* **198**, 385.e381-389 (2008).

44. D. L. Rolnik, D. Wright, L. C. Poon, N. O'Gorman, A. Syngelaki, C. de Paco Matallana, R. Akolekar, S. Cicero, D. Janga, M. Singh, . . . K. H. Nicolaides, Aspirin versus placebo in pregnancies at high risk for preterm preeclampsia. *N Engl J Med* **377**, 613-622 (2017).

45. E. Dimitriadis, D. L. Rolnik, W. Zhou, G. Estrada-Gutierrez, K. Koga, R. P. V. Francisco, C. Whitehead, J. Hyett, F. da Silva Costa, K. Nicolaides, E. Menkhorst, Pre-eclampsia. *Nat Rev Dis Primers* **9**, 8 (2023).

46. V. Agnello, D. Koffler, J. W. Eisenberg, R. J. Winchester, H. G. Kunkel, C1q precipitins in the sera of patients with systemic lupus erythematosus and other hypocomplementemic states: Characterization of high and low molecular weight types. *J Exp Med* **134**, 228-241 (1971).

47. S. Uwatoko, S. Aotsuka, M. Okawa, Y. Egusa, R. Yokohari, C. Aizawa, K. Suzuki, C1q solid-phase radioimmunoassay: Evidence for detection of antibody directed against the collagen-like region of c1q in sera from patients with systemic lupus erythematosus. *Clin Exp Immunol* **69**, 98-106 (1987).

48. M. Trendelenburg, J. Marfurt, I. Gerber, A. Tyndall, J. A. Schifferli, Lack of occurrence of severe lupus nephritis among anti-c1q autoantibody-negative patients. *Arthritis Rheum* **42**, 187-188 (1999).

49. F. S. van de Bovenkamp, D. J. Dijkstra, C. van Kooten, K. A. Gelderman, L. A. Trouw, Circulating c1q levels in health and disease, more than just a biomarker. *Mol Immunol* **140**, 206-216 (2021).

50. C. Liaskos, S. Rentouli, T. Simopoulou, A. Gkoutzourelas, G. Norman, A. Brotis, I. Alexiou, C. Katsiari, D. Bogdanos, L. Sakkas, Anti-c1q autoantibodies are frequently detected in patients with systemic sclerosis associated with pulmonary fibrosis. *British Journal of Dermatology* **181**, 138-146 (2019).

51. E. R. Volkmann, K. Andréasson, V. Smith, Systemic sclerosis. *Lancet* **401**, 304-318 (2023).

52. C. M. Wortel, S. I. Liem, N. M. van Leeuwen, M. Boonstra, C. M. Fehres, L. Stöger, T. W. Huizinga, R. E. Toes, J. De Vries-Bouwstra, H. U. Scherer, Anti-topoisomerase, but not anti-centromere b cell responses in systemic sclerosis display active, ig-secreting cells associated with lung fibrosis. *RMD Open* **9** (2023).

53. H. Vitkova, J. Jiskra, D. Springer, Z. Limanova, Z. Telicka, J. Bartakova, M. Trendelenburg, E. Potlukova, Anti-c1q autoantibodies are linked to autoimmune thyroid disorders in pregnant women. *Clin Exp Immunol* **186**, 10-17 (2016).

54. I. V. Menzhinskaya, L. V. Van'ko, M. M. Kashentseva, P. A. Kiryushchenkov, G. T. Sukhikh, Incidence of autoantibodies to c1q complement component in women with miscarriages and autoantibodies to phospholipids and chorionic gonadotropin. *Bull Exp Biol Med* **160**, 260-263 (2015).

55. A. Daponte, E. Deligeoroglou, S. Pournaras, C. Hadjichristodoulou, A. Garas, F. Anastasiadou, I. E. Messinis, Interleukin-15 (il-15) and anti-c1q antibodies as serum biomarkers for ectopic pregnancy and missed abortion. *Clin Dev Immunol* **2013**, 637513 (2013).

56. G. Moroni, A. Doria, E. Giglio, E. Imbasciati, C. Tani, M. Zen, F. Strigini, B. Zaina, A. Tincani, M. Gatto, F. de Liso, C. Grossi, P. L. Meroni, G. Cabiddu, P. Messa, P. Ravani, M. Mosca, Maternal outcome in pregnant women with lupus nephritis. A prospective multicenter study. *J Autoimmun* **74**, 194-200 (2016).

57. C. Agostinis, G. Zito, M. Toffoli, I. Peterlunger, L. Simoni, A. Balduit, E. Curtolo, A. Mangogna, B. Belmonte, D. Vacca, F. Romano, T. Stampalija, T. Salviato, F. Defendi, N. Di Simone, U. Kishore, G. Ricci, R. Bulla, A longitudinal study of c1q and anti-c1q autoantibodies in homologous and heterologous pregnancies for predicting pre-eclampsia. *Front Immunol* **13**, 1037191 (2022).

58. S. Uwatoko, S. Aotsuka, M. Okawa, Y. Egusa, R. Yokohari, C. Aizawa, K. Suzuki, Characterization of c1q-binding igg complexes in systemic lupus erythematosus. *Clin Immunol Immunopathol* **30**, 104-116 (1984).

59. F. J. Beurskens, R. A. van Schaarenburg, L. A. Trouw, C1q, antibodies and anti-c1q autoantibodies. *Molecular immunology* **68**, 6-13 (2015).

60. I. E. Coremans, M. R. Daha, E. A. van der Voort, C. E. Siegert, F. C. Breedveld, Subclass distribution of iga and igg antibodies against clq in patients with rheumatic diseases. *Scand J Immunol* **41**, 391-397 (1995).

61. Q. Y. Fang, F. Yu, Y. Tan, L. X. Xu, L. H. Wu, G. Liu, F. M. Shao, M. H. Zhao, Anti-c1q antibodies and igg subclass distribution in sera from chinese patients with lupus nephritis. *Nephrol Dial Transplant* **24**, 172-178 (2009).

62. L. A. Trouw, T. W. Groeneveld, M. A. Seelen, J. M. Duijs, I. M. Bajema, F. A. Prins, U. Kishore, D. J. Salant, J. S. Verbeek, C. van Kooten, M. R. Daha, Anti-c1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular c1q-containing immune complexes. *J Clin Invest* **114**, 679-688 (2004).

63. E. C. So, H. Zhou, A. Greenwell, E. E. Burch, Y. Ji, E. Y. Mérigeon, H. S. Olsen, S. M. Bentzen, D. S. Block, X. Zhang, S. E. Strome, Complement component c1q is an immunological rheostat that regulates fc:Fcγr interactions. *Immunogenetics* **75**, 369-383 (2023).

64. Y. Deng, Y. Zheng, D. Li, Q. Hong, M. Zhang, Q. Li, B. Fu, L. Wu, X. Wang, W. Shen, Y. Zhang, J. Chang, K. Song, X. Liu, S. Shang, G. Cai, X. Chen, Expression characteristics of interferon-stimulated genes and possible regulatory mechanisms in lupus patients using transcriptomics analyses. *EBioMedicine* **70**, 103477 (2021).

65. A. Norvell, L. Mandik, J. G. Monroe, Engagement of the antigen-receptor on immature murine b lymphocytes results in death by apoptosis. *J Immunol* **154**, 4404-4413 (1995).

66. F. A. Bonilla, H. C. Oettgen, Adaptive immunity. *J Allergy Clin Immunol* **125**, S33-40 (2010).

APPENDICES

English summary

The immune system is an important factor in the protection against disease and infection, and it consists of an innate part and an adaptive part. The innate part recognizes several common molecules and patterns associated with infection and damage, and rapidly reacts to such signals. Cells of the adaptive immune system can be very specific towards one molecule, but need more time to develop and mature in a primary immune response. Notably, in the adaptive immune response, memory cells are formed that shorten the reaction time when their particular target is encountered again. Antibodies are produced by a specific cell type in the adaptive immune system, called B cells. These antibodies exist as various types and specifically bind a target with their antigen-binding region. When bound to their target, they can activate other elements of the immune system as an effector mechanism. One of these elements is the complement system, which is a group of proteins in the innate immune system that can activate each other in a highly regulated chain reaction. Complement activation starts with the classical, lectin or alternative pathway depending on the activator. The initiator molecule of the classical pathway is called C1, which consists of recognition protein C1q and enzymes C1r and C1s. C1q is capable of recognizing several different structures, such as bacterial proteins, dying cells, and target-bound antibodies.

The immune system is critical in the defense of the human body against pathogens. However, as introduced in **chapter 1**, the immune system sometimes inadvertently turns against the body it should be protecting; this is called autoimmunity. This situation may arise through genetic disorders causing over-activation of the immune system, or when cells of the adaptive part of the immune system start recognizing structures that belong to the body itself, so-called self-antigens. When this recognition of self-antigens occurs in B cells and is not halted, it leads to the production of antibodies against self-antigens, called autoantibodies. Various types of autoantibody are associated with different autoimmune diseases, highlighting the relevance of these antibodies. In this thesis, discussion of autoantibodies mainly focuses on those targeting the initiator of the classical pathway of complement activation, C1q. An interesting feature of anti-C1q autoantibodies is their specificity to solid-phase C1q, a conformational state which C1q adopts upon binding to its ligands. This type of autoantibody is strongly associated with systemic lupus erythematosus (SLE) and especially lupus nephritis. Due to the crucial role of autoantibodies as well as the complement system in (auto)immunity, this thesis describes C1q and anti-C1q autoantibodies in the context of several diseases.

In **chapter 2**, we reviewed the role the complement system plays in several rheumatic diseases. The role of complement in the pathogenesis of rheumatoid arthritis (RA) is

mainly ascribed to the alternative pathway, while the classical pathway only plays a minor role. In the meantime, the influence of complement on the rheumatic disease SLE can be two-fold. Firstly, although rare, deficiency of C1q almost always leads to development of SLE. Secondly and more commonly, SLE severity is correlated with large-scale complement activation and deposition in tissues. Based on the evidence, one would expect complement to play a large role in the processes that drive RA and SLE. Complement-inhibiting drugs (including the anti-C5 therapeutic antibody eculizumab) were tested, but were deemed to have insufficient impact on both diseases. Conversely, in the case of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) there was initially no suspicion for a large role of complement. This changed when stimulation of neutrophils through their C5a receptor was shown to be critical for the disease process. Subsequent research and development led to the approval and marketing of avacopan, a compound blocking the C5a receptor. While complement activation is observed in many rheumatic diseases, therapeutic inhibition of complement was shown to only be effective in some diseases.

Tuberculosis (TB) can occur as active disease or in a latent form. The diagnosis of active TB disease continues to prove difficult, as current tests either do not distinguish between latent and active infection, or take a long time to reach conclusion. This is a problem for pulmonary TB as well as extrapulmonary manifestations of TB, such as TB-associated uveitis. In **chapter 3**, we confirmed the value of C1q as a biomarker for active pulmonary TB disease and also established the association between high serum C1q concentration and TB uveitis. Furthermore, we observed an inverse correlation between high C1q concentration and expression of a previously published signature set of interferon-stimulated genes. The combination of serum C1q concentration and the interferon signature could almost fully separate healthy individuals from patients with active pulmonary TB disease, therefore this combination may be useful for future clinical diagnostics.

Chapter 4 describes our research into complement and anti-complement autoantibodies during pregnancy and in the pregnancy complication preeclampsia. Here, we observed that serum concentrations of both C1q and factor H were higher in women with a healthy pregnancy than in nonpregnant controls. Possibly, C1q concentration is increased to fulfill its role in the construction of new blood vessels and in tissue remodeling, for which there is great need in the placenta. On the other hand, factor H is a complement regulator and may be useful to limit complement activation in the remodeling placenta. Autoantibodies to C1q and factor H were also investigated in the same chapter, but presence of these antibodies was not significantly different between preeclampsia and control pregnancy

groups. We observed lower serum factor H concentration in women with preeclamptic pregnancies than in women with control pregnancies. This difference was driven by early-onset cases of preeclampsia, as factor H concentration was lower in these cases compared to late-onset cases. These results indicate a role for C1q and factor H during pregnancy, and suggest that low factor H concentration may be involved in the pathogenesis of preeclampsia.

The rheumatic disease systemic sclerosis involves fibrosis of the skin and internal organs. Pulmonary fibrosis and pulmonary arterial hypertension are the most common lung-associated conditions in systemic sclerosis, contributing highly to mortality. In **chapter 5**, we investigated the presence of anti-C1q autoantibodies among systemic sclerosis patients, in order to find a prognostic marker for these conditions. We found that anti-C1q was not present at a higher rate in systemic sclerosis patients than in healthy controls. Moreover, lung fibrosis and pulmonary arterial hypertension were not increased in patients that did have anti-C1q autoantibodies, contrary to an earlier report. Therefore we concluded that anti-C1q autoantibodies would not have a good prognostic value for lung conditions in systemic sclerosis patients. Furthermore, the observed association of anti-C1q with anti-topoisomerase antibodies, which was already reported to associate with lung complications, would detract from any added value of anti-C1q in systemic sclerosis prognostics.

In **chapter 6**, we characterized several human anti-C1q autoantibodies. We isolated B cells that produce anti-C1q antibodies from healthy donors, and subsequently produced these antibodies recombinantly. This allowed for the molecular characterization of 9 monoclonal human anti-C1q antibodies. The anti-C1q autoantibodies were of the immunoglobulin G (IgG) isotype and contained variable domains with a considerable number of mutations from the germline sequence. We confirmed the specificity for solid-phase C1q, and measured binding of anti-C1q to C1q while C1q was bound on a range of its natural ligands such as IgG, IgM, CRP and necrotic cells. We observed that the antibodies bound specifically to the collagen-like region of C1q and competition experiments reveal that at least 2 distinct epitopes are targeted by human anti-C1q. Electron microscopy narrowed down the location of at least one of these epitopes to be in a region close to the globular heads of C1q, and further showed that this epitope is present multiple times on a C1q molecule.

The anti-C1q autoantibodies isolated in **chapter 6** showed competition for binding to C1q with anti-C1q in the serum of SLE patients, indicating that they bind the same or similar epitopes on C1q. Addition of anti-C1q antibodies to C1q-containing immune

complexes did not yield additional complement activation, but did increase binding of Fcγ-receptors. In cellular assays, this translated into the ability of anti-C1q to enhance the phagocytosis of C1q-opsonized particles by human phagocytes, demonstrating the capacity to functionally engage Fc receptors. These data led us to propose a model for the involvement of anti-C1q autoantibodies in lupus nephritis, a disease they are strongly associated with. We know from literature that deposited immune complexes are necessary for anti-C1q to be pathogenic. These immune complexes bind C1q and activate complement. According to our hypothesis, complement activation attracts immune cells through anaphylatoxins, however Fc receptors on these cells are not yet engaged because C1q masks the antibodies in the immune complex. Only when anti-C1q autoantibodies bind to deposited, C1q-containing complexes, do the immune cells have a target for their Fc receptors, leading to cellular activation and damage to the kidney of the SLE patient. In healthy individuals, such as the donors from whom we isolated the anti-C1q antibodies, no immune complexes are deposited in the kidney, therefore they do not develop nephritis even if they have anti-C1q autoantibodies.

Together, the research described in this thesis shows that complement, and mainly C1q, confers pathogenic effects, contributes to physiology, is a therapeutic target and a diagnostic tool, all besides its traditional role in fighting infections. Additionally, studies characterizing autoantibodies against C1q allowed for new understanding of their molecular features and immunological impact. The research presented here therefore urges further investigation into C1q and anti-C1q autoantibodies, and hopefully provides a foundation for future clinical application.

Nederlandse samenvatting

Het immuunsysteem kan worden opgedeeld in een aangeboren deel en een verworven deel, die samen zorgen voor bescherming tegen ziektes. Het aangeboren deel herkent een aantal patronen die aanwezig zijn op veel ziekteverwekkers. Hierdoor kan het aangeboren immuunsysteem zeer snel reageren op een nieuwe infectie, maar is het niet zo specifiek. Het verworven immuunsysteem is daarentegen zeer specifiek, maar heeft meer tijd nodig om een reactie te ontwikkelen. Bovendien blijft de specificiteit van het verworven immuunsysteem gedeeltelijk in stand tot wel tientallen jaren nadat het heeft gereageerd op bijvoorbeeld een infectie of vaccinatie. Zo kunnen deze cellen als 'geheugen' dienen en sneller reageren als ze hun specifieke doelwit opnieuw tegenkomen. Een belangrijk onderdeel van het verworven immuunsysteem zijn B cellen, die verantwoordelijk zijn voor het maken van antilichamen. Deze moleculen spelen een cruciale rol in de afweer tegen infecties door heel specifieke moleculen (op bijvoorbeeld bacteriën en virussen) te herkennen en vervolgens ofwel af te dekken, of andere delen van het immuunsysteem te activeren tegen dit doelwit. Antilichamen komen voor in verschillende types, die elk via andere effector mechanismen het immuun systeem kunnen activeren.

Een van de delen van het aangeboren immuunsysteem dat door antilichamen geactiveerd kan worden is het complementsysteem. Dit is een groep van enkele tientallen verschillende moleculen die aanwezig zijn in het bloed, en die op verschillende manieren geactiveerd kunnen worden. Het complementsysteem is onderverdeeld in een klassieke, lectine en alternatieve route. De moleculen in iedere route kunnen steeds een volgend molecuul in een kettingreactie activeren, totdat ze samenkomen bij de moleculen C3 en C5. Vanaf daar volgt de kettingreactie voor alle routes hetzelfde pad, waarbij een complex van moleculen samenkomt en een gat kan vormen in het membraan van de cel waar ze aan gebonden zijn. Daarnaast kunnen verschillende geactiveerde complementmoleculen binden aan receptoren op cellen van het immuunsysteem, om deze aan te trekken en aan te zetten tot actie. Het herkennen van doelwitten wordt in de klassieke route van het complementsysteem gedaan door C1q, een molecuul waar een groot deel van dit proefschrift over gaat. Binding van C1q aan ziekteverwekkers kan plaatsvinden doordat C1q direct aan structuren van de pathogeen bindt, of doordat er eerst antilichamen aan de pathogeen binden waaraan vervolgens C1q kan binden. Directe binding van C1q aan dode lichaamseigen cellen speelt daarnaast een belangrijke rol bij het tijdig verwijderen van deze cellen.

Een belangrijk proces in alle delen van het immuunsysteem is het onderscheiden van lichaamseigen en lichaamsvreemde moleculen. Zoals geïntroduceerd in **hoofdstuk 1** gaat

het maken van dit onderscheid helaas wel eens mis en valt het immuunsysteem het eigen lichaam aan; dit noemen we auto-immuniteit. Dit kan veroorzaakt worden door factoren uit de omgeving, genetische afwijkingen die zorgen voor een te actief immuunsysteem, of door cellen van het verworven immuunsysteem die een onderdeel van het eigen lichaam beginnen te herkennen als doelwit. Wanneer deze herkenning van lichaamseigen structuren door B cellen niet geremd wordt, leidt dat tot de productie van antilichamen tegen het eigen lichaam, zogenoemde autoantilichamen. In dit proefschrift worden vooral autoantilichamen beschreven die binden aan C1q. Deze anti-C1q antilichamen komen voor bij een paar procent van de algemene bevolking, maar zijn vooral bekend vanwege hun relatie met de auto-immuunziekte Systemische Lupus Erythematodes (SLE) en specifiek de bijbehorende nieraandoening lupus nefritis. Anti-C1q autoantilichamen herkennen C1q alleen als C1q in een specifieke vorm aanwezig is. Wanneer C1q bindt aan een structuur verandert het een beetje van vorm, wat ook een deel van C1q nieuw beschikbaar maakt wat eerder verborgen was. Deze vorm van C1q noemen we ligand-gebonden C1q. Specifiek het deel van C1q dat in deze vorm beschikbaar komt, wordt herkend door anti-C1q autoantilichamen. Om deze reden kunnen C1q en anti-C1q tegelijk in het bloed voorkomen zonder aan elkaar te binden; dat gebeurt pas nadat C1q een ligand vindt en van vorm verandert. Vanwege het belangrijke aandeel van autoantilichamen en ook het complementsysteem in gezondheid en auto-immuniteit, beschrijft dit proefschrift onderzoeken naar C1q en anti-C1q autoantilichamen in de context van verschillende ziektes. Daarbij komt eerst een literatuuronderzoek aan bod over complement in reumatische ziektes, vervolgens hoofdstukken over C1q in tuberculose en zwangerschap en tot slot autoantilichamen tegen C1q.

In **hoofdstuk 2** maakten we een overzicht van de rol van complement in verschillende reumatische ziektes. Voor reumatoïde artritis (RA) wordt de rol van complement vooral toegeschreven aan de alternatieve route, terwijl de klassieke route een kleinere rol speelt. In het geval van SLE is de invloed van complement tweezijdig. In het zeldzame geval dat iemand helemaal geen C1q heeft, krijgt diegene bijna altijd SLE. Wat vaker voorkomt is dat ernstige SLE samengaat met grootschalige activatie en neerslag van complement in organen en weefsels. Dit gaat soms zo ver dat een groot deel van de complementmoleculen worden opgebruikt. Op basis van deze feiten zou men verwachten dat complement een grote rol speelt in de ziekteprocessen die RA en SLE veroorzaken. Daarom zijn er verschillende medicijnen zoals eculizumab, een antilichaam dat de activatie van C5 in de kettingreactie stopt, getest in patiënten met deze ziektes. Echter bleek na onderzoek dat deze medicijnen onvoldoende invloed hadden op de ernst van RA en SLE. In een andere auto-immuunziekte, genaamd anti-neutrofiel cytoplasmatisch antilichaam geassocieerde vasculitis (in het

Engels afgekort tot AAV), werd in eerste instantie juist niet verwacht dat complement een grote rol zou spelen. Dit veranderde echter toen men erachter kwam dat aantrekking en stimulatie van neutrofielen (een type immuuncel) door complementmolecuul C5a een cruciaal onderdeel was van het ziekteproces van AAV. Vervolgens heeft verder onderzoek geleid tot de ontwikkeling en goedkeuring van avacopan, een molecuul dat de receptor voor C5a blokkeert zodat neutrofielen minder sterk worden geactiveerd. Activatie van het complementsysteem wordt dus in veel reumatische ziektes gezien, maar het blokkeren van complement met medicijnen is slechts bij enkele van deze ziektes effectief.

Tuberculose (TBC) kan voorkomen als een actieve ziekte, of in latente vorm. De diagnose van TBC is nog altijd lastig te stellen, omdat huidige testen geen onderscheid maken tussen de actieve en de latente vorm, of erg lang duren alvorens een conclusie kan worden getrokken. Dit is vooral een probleem in landen waar TBC veel voorkomt en dus een aanzienlijk deel van de bevolking een latente tuberculose-infectie heeft. TBC komt meestal voor in de longen, maar kan ook andere organen infecteren, waaronder de ogen (genaamd TBC uveitis). Hierbij is diagnose met de huidige testen nog lastiger vanwege de geringe bereikbaarheid. In **hoofdstuk 3** konden we de waarde van C1q als biomarker bevestigen om actieve TBC in de longen aan te tonen en vaststellen dat er een verband is tussen de concentratie van C1q in serum en de aanwezigheid van TBC uveitis. Daarnaast vonden we een negatieve correlatie tussen hoge C1q concentratie in serum enerzijds, en de transcriptie van een eerder gepubliceerde groep genen die door interferon gestimuleerd worden anderzijds. Door te kijken naar de combinatie van de C1q concentratie en de transcriptie van interferon-gestimuleerde genen, konden we gezonde mensen bijna volledig onderscheiden van patiënten met actieve TBC in de longen. Op deze manier kunnen deze testen mogelijk in de toekomst deel gaan uitmaken van TBC diagnostiek.

Hoofdstuk 4 beschrijft ons onderzoek naar complement en autoantilichamen tegen complement tijdens zwangerschap en de complicatie pre-eclampsie, ook wel zwangerschapsvergiftiging. Dit is een aandoening die tijdens de tweede helft van de zwangerschap aan het licht komt bij 3% tot 5% van de zwangerschappen en die zich kenmerkt door eiwitverlies in de urine en een te hoge bloeddruk. Deze aandoening stopt pas weer na de bevalling. In dit onderzoek hebben we de concentraties C1q en factor H, een remmer van het complementsysteem, gemeten. Dit deden we in serummonsters van niet zwangere vrouwen, vrouwen met een gezonde zwangerschap en vrouwen met pre-eclampsie tijdens hun zwangerschap, afkomstig uit Nederland, Finland en Noorwegen. In het eerste deel zagen we een hogere concentratie van zowel C1q als factor H in het serum van zwangere vrouwen vergeleken met niet zwangere vrouwen.

Mogelijk wordt dit met opzet door het lichaam gedaan, omdat C1q een rol speelt bij de aanleg van nieuwe bloedvaten en het modelleren van weefsel, beide processen die nodig zijn in een placenta. Factor H kan daarentegen activatie van complement remmen en zou zo onbedoelde schade kunnen voorkomen tijdens de aanleg van de placenta. In **hoofdstuk 4** onderzochten we ook autoantilichamen tegen C1q en factor H, maar de aanwezigheid hiervan verschilde niet tussen vrouwen met en zonder pre-eclampsie. De concentratie van factor H was echter lager in vrouwen met pre-eclampsie dan in vrouwen met een gezonde zwangerschap. Het verschil tussen deze groepen werd voornamelijk veroorzaakt door de gevallen van pre-eclampsie met vroege aanvang, waarbij de bevalling plaatsvindt voor de 34^{e} week van de zwangerschap voorbij is. De resultaten van dit onderzoek suggereren een rol voor C1q en factor H tijdens zwangerschap, en brengt een verlaagde concentratie factor H in verband met de ziekte pre-eclampsie.

De reumatische ziekte systemische sclerose, die we in **hoofdstuk 5** hebben onderzocht, kenmerkt zich door fibrose, een soort littekenvorming van de huid en interne organen. Dit komt bij veel patiënten ook voor in de longen, waar we het longfibrose noemen, wat bijdraagt aan de ernst van deze aandoening. Een eerder onderzoek bracht autoantilichamen tegen C1q in verband met longfibrose in patiënten met systemische sclerose. Om een betere voorspelling te kunnen maken van welke patiënten longfibrose krijgen, zijn we ook anti-C1q gaan meten in patiënten met systemische sclerose in het Leids Universitair Medisch Centrum. Echter kwamen we tot de conclusie dat anti-C1q autoantilichamen geen goede voorspellende waarde hebben voor longfibrose in systemische sclerose. Bovendien zagen we dat de meerderheid van systemische sclerose patiënten met anti-C1q ook al autoantilichamen had tegen het molecuul topoisomerase. Deze autoantilichamen zijn op zichzelf al geassocieerd met longaandoeningen binnen systemische sclerose, wat helaas af zou doen aan enige toegevoegde waarde van anti-C1q metingen voor systemische sclerose patiënten.

In **hoofdstuk 6** beschreven we het onderzoek naar de moleculaire karakteristieken van anti-C1q autoantilichamen. Hierbij hebben we specifiek de B cellen die anti-C1q produceren geïsoleerd uit gezonde donoren en het genetisch materiaal hier uitgehaald. Daarmee konden we in totaal 9 verschillende anti-C1q antilichamen namaken en onderzoeken. Tot nu toe waren vooral antilichamen in serum van donoren onderzocht, met als nadeel dat men altijd een mix van verschillende antilichamen onderzocht zonder de exacte samenstelling of concentratie te weten. De antilichamen waren allemaal van het IgG type (het meest voorkomende type in menselijk bloed) en hadden een aanzienlijk aantal mutaties in het variabele deel. Dit betekent dat de B cellen die deze antilichamen produceren een behoorlijke ontwikkeling hebben doorgemaakt, waarschijnlijk met hulp

en stimulatie van andere immuuncellen. Verder hebben we bevestigd dat de anti-C1q autoantilichamen inderdaad specifiek zijn voor ligand-gebonden C1q en hebben we binding van anti-C1q aan C1q gemeten terwijl C1q gebonden was aan verschillende natuurlijke liganden, zoals antilichamen, CRP en dode cellen.

Experimenten om competitie te meten tussen antilichamen lieten zien dat onze anti-C1q autoantilichamen op te delen zijn in 2 groepen, waarbij elke groep aan een ander deel van C1q bindt. C1q bestaat uit 6 koppen die elk een collageenachtige staart hebben. De staarten zitten aan elkaar vast en vormen samen de collageenachtige regio. In onze experimenten zagen we dat alle anti-C1q autoantilichamen aan de collageenachtige regio van C1q binden. Door middel van elektronenmicroscopie toonden we aan dat een van de antilichamen dichtbij de koppen van C1q bindt, en tevens dat meerdere van deze identieke antilichamen tegelijk aan hetzelfde C1q molecuul kunnen binden. De antilichamen die we in **hoofdstuk 6** hebben nagemaakt waren afkomstig van gezonde donoren, echter is het ook interessant om te weten wat we op basis van deze antilichamen kunnen leren over anti-C1q autoantilichamen in SLE patiënten. Daarom voerden we opnieuw experimenten uit om competitie te bekijken, dit keer tussen onze geïsoleerde anti-C1q en anti-C1q in het serum van SLE patiënten. Hieruit bleek dat beide groepen anti-C1q binden op dezelfde of vergelijkbare plekken op C1q.

Wanneer antilichamen binden aan hun doelwit, kunnen ze verschillende andere delen van immuunsysteem activeren, afhankelijk van het type antilichaam. Antilichamen van het type IgG1, zoals onze nagemaakte anti-C1q, kunnen zowel C1q binden om complement te activeren alsook om Fc receptoren op immuuncellen activeren. In hoeverre beide ook daadwerkelijk gebeurt hangt af van verschillende omstandigheden, zoals de manier waarop het antilichaam aan zijn doelwit bindt. Daarom onderzochten we welke mechanismen geactiveerd worden wanneer anti-C1q aan ligand-gebonden C1q bindt. De toevoeging van anti-C1q aan ligand-gebonden C1q zorgde niet voor extra activatie van complement, maar wel voor binding en activatie van immuuncellen via Fc receptoren. Op basis van deze gegevens konden we een hypothese opstellen voor de rol van anti-C1q in het ziekteproces van nierontsteking in SLE patiënten (lupus nefritis). Uit de literatuur is bekend dat immuuncomplexen (dat zijn clusters van antilichamen en hun doelwitten) in de nieren cruciaal zijn voor de ziekmakende invloed van anti-C1q. Bovendien zijn zowel complement activatie als Fc receptoren nodig. Aan de immuuncomplexen bindt C1q, wat zorgt voor complement activatie. Volgens onze hypothese zorgt deze complement activatie niet meteen voor de schade aan de nier, maar geactiveerde stukjes van complementmoleculen trekken wel immuuncellen aan. De Fc receptoren op deze immuuncellen kunnen echter niet aan de immuuncomplexen

binden, omdat C1q daar al op zit en de bindingsplaats blokkeert. Wanneer anti-C1q autoantilichamen vervolgens aan C1q binden, kunnen Fc receptoren op hun beurt aan anti-C1q antilichamen binden. Hierdoor raken de immuuncellen met Fc receptoren geactiveerd en scheiden ze schadelijk stoffen uit, die de nieren beschadigen. Dit hele proces is echter afhankelijk van de aanwezigheid van immuuncomplexen die neerslaan in de nier; in SLE patiënten komen deze immuuncomplexen vaak voor. Gezonde personen, zoals de donoren waaruit wij anti-C1q autoantilichamen hebben geïsoleerd, hebben normaalgesproken vrijwel geen immuuncomplexen in hun bloed, waardoor ze geen nierontsteking ontwikkelen ondanks de anti-C1q autoantilichamen.

Het in dit proefschrift beschreven onderzoek laat zien dat complement, en met name C1q, naast zijn traditionele rol in de afweer tegen infecties ook ziekmakende effecten heeft, bijdraagt aan gezonde fysiologie en een therapeutisch doelwit en een diagnostisch hulpmiddel is. Daarnaast hebben de studies naar anti-C1q autoantilichamen geleid tot nieuw inzicht in hun moleculaire kenmerken en immunologische invloed. Met dit werk hopen we aanleiding te geven tot verder onderzoek naar C1q en anti-C1q autoantilichamen, en een basis te vormen voor toekomstige klinische toepassing.

List of publications

DJ Dijkstra, FS van de Bovenkamp, L Abendstein, R Zuijderduijn, J Pool, CSM Kramer, L Slot, JW Drijfhout, L de Vor, KA Gelderman, SHM Rooijakkers, A Zaldumbide, G Vidarsson, TH Sharp, PWHI Parren, LA Trouw. **Human anti-C1q autoantibodies bind specifically to solid-phase C1q and enhance phagocytosis but not complement activation.** PNAS. 2023 doi: 10.1073/pnas.2310666120

DJ Dijkstra, AI Lokki, LM Gierman, NV Borggreven, C van der Keur, M Eikmans, KA Gelderman, H Laivuori, FINNPEC Core Investigator Group, A-C Iversen, MLP van der Hoorn, LA Trouw. **Circulating Levels of Anti-C1q and Anti-Factor H Autoantibodies and Their Targets in Normal Pregnancy and Preeclampsia.** Front Immunol. 2022 doi: 10.3389/fimmu.2022.842451

DJ Dijkstra, SIE Liem, NM van Leeuwen, CM Fehres, JK de Vries-Bouwstra, LA Trouw. **Anti-C1q autoantibodies may not serve as an adequate biomarker for lung manifestations in systemic sclerosis: a single-centre, cross-sectional study.** Br J Dermatol. 2021 doi: 10.1111/bjd.20412

B Schrijver*, DJ Dijkstra*, NV Borggreven, R La Distia Nora, E Huijser, MA Versnel, PM van Hagen, SA Joosten, LA Trouw, WA Dik. **Inverse correlation between serum complement component C1q levels and whole blood type-1 interferon signature in active tuberculosis and QuantiFERON-positive uveitis: implications for diagnosis.** Clin Transl Immunology. 2020 doi: 10.1002/cti2.1196 (*Shared first author)

DJ Dijkstra, JV Joeloemsingh, IM Bajema, LA Trouw. **Complement activation and regulation in rheumatic disease.** Semin Immunol. 2019 doi: 10.1016/j.smim.2019.101339

L Abendstein, DJ Dijkstra, RTN Tjokrodirijo, PA van Veelen, LA Trouw, PJ Hensbergen, TH Sharp. **Complement is activated by elevated IgG3 hexameric platforms and deposits C4b onto distinct antibody domains.** Nat Commun. 2023 doi: 10.1038/s41467-023-39788-5

P Niewold, DJ Dijkstra, Y Cai, D Goletti, F Palmieri, KE van Meijgaarden, FAW Verreck, OW Akkerman, RW Hofland, EM Delemarre, S Nierkens, MK Verheul, AJ Pollard, JT van Dissel, THM Ottenhoff, LA Trouw, SA Joosten. **Identification of circulating monocytes as producers of tuberculosis disease biomarker C1q.** Sci Rep. 2023 doi: 10.1038/s41598-023-38889-x

L Abendstein, WEM Noteborn, LS Veenman, DJ Dijkstra, FS van de Bovenkamp, LA Trouw, TH Sharp. **DNA nanostructure-templated antibody complexes provide insights into the geometric requirements of human complement cascade activation.** bioRxiv preprint 2023 doi: 10.1101/2023.10.20.562751

DP Noone, DJ Dijkstra, TT van der Klugt, PA van Veelen, AH de Ru, PJ Hensbergen, LA Trouw, TH Sharp. **PTX3 structure determination using a hybrid cryoelectron microscopy and AlphaFold approach offers insights into ligand binding and complement activation.** PNAS. 2022 doi: 10.1073/pnas.2208144119

FS van de Bovenkamp, DJ Dijkstra, C van Kooten, KA Gelderman, LA Trouw. **Circulating C1q levels in health and disease, more than just a biomarker.** Mol Immunol. 2021 doi: 10.1016/j.molimm.2021.10.010

R Lubbers, SC Oostindie, DJ Dijkstra, PWHI Parren, MK Verheul, L Abendstein, TH Sharp, A de Ru, GMC Janssen, PA van Veelen, ETJ van den Bremer, B Bleijlevens, B-J de Kreuk, FJ Beurskens, LA Trouw. **Carbamylation reduces the capacity of IgG for hexamerization and complement activation.** Clin Exp Immunol. 2020 doi: 10.1111/cei.13411

R Lubbers, JS Sutherland, D Goletti, RA de Paus, DJ Dijkstra, CHM van Moorsel, M Veltkamp, SMT Vestjens, WJW Bos, L Petrone, ST Malherbe, G Walzl, KA Gelderman, GH Groeneveld, A Geluk, THM Ottenhoff, SA Joosten, LA Trouw. **Expression and production of the SERPING1-encoded endogenous complement regulator C1-inhibitor in multiple cohorts of tuberculosis patients.** Mol Immunol. 2020 doi: 10.1016/j.molimm.2020.02.006

Curriculum vitae

Douwe Jan Dijkstra was born on Tuesday the 3rd of August 1993 in Groningen and largely grew up in Elst. From 2005 to 2011 he attended secondary school at Overbetuwe College in Bemmel, where he completed pre-university education. Douwe subsequently started the BSc program Biotechnology at Wageningen University, graduating in 2014. During his BSc internship, performed under supervision of Prof. Dr. Gorben Pijlman and Dr. Mia Hikke at the Virology department of Wageningen University, he studied the temperature-dependent production of Salmonid Alphavirus glycoproteins. Douwe continued with the MSc Medical Biotechnology, in which he incorporated a 4-month exchange to the Technical University of Denmark to follow courses in their Pharmaceutical Design and Engineering program. His first MSc internship took place at the Nematology department of Wageningen University, where he produced and investigated allergy-inducing proteins under supervision of Dr. Arjen Schots and Dr. Kim van Noort. A second MSc internship followed, performed at Genmab in Utrecht with guidance from Dr. Aran Labrijn and Dr. Frank Beurskens. During this time, Douwe studied the role of the IgG hinge in complement activation and developed a great interest in the function and application of antibodies. Soon after graduating his MSc Medical Biotechnology in 2017, he started the PhD research described in this thesis under supervision of Prof. Dr. Leendert Trouw and Prof. Dr. Paul Parren at the Immunology department of the Leiden University Medical Center (LUMC). In 2021, Douwe transitioned to the role of researcher within the same department, for a two-year project focused again on the study of complement activation by engineered antibodies in a collaborative project with industry partner Genmab. During his time at the LUMC, Douwe supervised 4 student internships and was able to present his work in 5 oral presentations to fellow scientists at national and international meetings.

Dankwoord

Het werk zit er op, wat een goed gevoel! Maar meteen ook de gedachte dat ik hiervoor zoveel mensen dank ben verschuldigd, voor hun steun bij alle experimenten, het schrijven van artikelen en vaak ook mentale ondersteuning. Met elk jaar van mijn promotietraject kwamen er mensen bij om te bedanken, dus ik heb nogal een lijst.

Allereerst Leendert, mijn promotor en mentor tijdens al die jaren, bedankt voor alles wat ik van je heb geleerd, je hebt me gestimuleerd alles uit mezelf te halen en gestuurd wanneer ik dat nodig had. Ik heb me altijd prettig gevoeld bij de goede sfeer die je in het team hield (ondanks wisselende samenstellingen, corona-lockdowns en een ski-ongeluk), mede daardoor heb ik vele positieve herinneringen gemaakt en ben ik ook door mijn mindere momenten heen gekomen.

Bedankt ook aan Paul, mijn andere promotor, voor je inzichten, inzet voor het onderzoek en jouw kritische blik op wat ik schreef. Dit heb ik zeer gewaardeerd en ik heb veel van je geleerd, ondanks dat je vaak op afstand was. Tevens dank aan mijn promotiebegeleidingscommissie, Sebastiaan en René, voor hun hulp en adviezen.

Daarnaast is mijn dank groot voor alle collega's in het complement-team. Sanne, Jos, Rob, Nicole, Michelle, Lu, Stef (mijn ervaren paranimf), Haiyu, Saskia, Rick, Karin and Thika thank you for all your help in the lab, the fantastic team spirit inside and outside the office and allowing me to turn up the volume whenever there was a quiz or the 'foute uur' on the radio. You were a great reason to enjoy my time in the LUMC. Ook mijn studenten Joep, Teun, Jonas en Myrthe, bedankt voor jullie hulp aan mijn projecten. Ik heb veel geleerd van alle vragen die jullie stelden en het gaf me voldoening jullie te helpen in je ontwikkeling als onderzoeker.

From all 4 different offices I occupied at the LUMC, I want to thank my roommates Moniek, Cynthia, Caroline, Sanne, Michelle, Anita, May, Helena, Hanneke, Carin, Juliette, Suzanne, Snow, Ilse, Gonca, Lu, Felix, Jasper, Nils, and Fréderique for their advice, distraction and great working atmosphere. This extends to the rest of the Immunology department, thank you for your support during my time here.

All my colleagues elsewhere in the LUMC, thank you for your contributions to the research in my thesis. A special word of thanks to Thom, Leoni, Dylan and Sebastiaan for productive collaborations, interesting discussions and good memories.

Ik neem hierbij ook de kans om al mijn stagebegeleiders te bedanken, zij hebben mij laten zien hoe het is je eigen onderzoek uit te voeren. En dan speciaal Frank en Aran, die mijn interesse in antilichamen hebben gevoed.

To the LIFI course club, Mikhail, Mikołaj, Annieck, and Kyra, thanks for making a place to share the ups and downs of PhD life and I hope we can continue this after we get our PhDs.

Mijn vrienden van The Meeting: Sjors, Marijke, Bas, Elianne, Jan, Fleur, Lisanne, Alex, Nils, Anne, Eva, Aniek, Ruben, Fleur, Dyon, Hannah en Mark, we zijn samen opgegroeid en ik ben blij dat we elkaar nog steeds zien. Bedankt voor jullie vriendschap, interesse en afleiding tijdens avonden en weekenden wanneer ik dat nodig had, zowel tijdens mijn promotietijd als daarvoor. Aira, Débora and Mariela, thank you for inviting me into your group. En natuurlijk dankjewel aan mijn Wageningse vrienden Walter, Els, Daphne, Wiegert en Iris voor leuke spellen, een luisterend oor en hechte vriendschap.

Dank aan mijn familie, voor interesse in mijn werk en voor de omgeving waarin ik opgroeide. Mijn (aanstaande) schoonfamilie Jan, Léonie, Yvonne en Joris, bedankt voor jullie steun en de gezellige momenten samen. Mijn kleine broertje Jelle, je bent een geweldige broer en goede vriend. Ik waardeer je om hoe we zo anders en toch hetzelfde zijn. Wat fijn dat jij straks naast me staat als paranimf! Mijn ouders, Arjen en Gea, jullie ben ik enorm dankbaar voor jullie opvoeding die me gemaakt heeft wie ik nu ben. Jullie hebben me altijd ondersteund, geholpen en geadviseerd waar ik dat nodig had, maar me ook losgelaten om op eigen benen te leren staan. Ik had me niets meer kunnen wensen.

Mijn lieve verloofde Marloes, tegelijk ons promotietraject doorlopen was niet altijd makkelijk, maar wat ben ik gelukkig met jou in mijn leven. Bedankt voor alle momenten waarop je me opvrolijkt, motiveert, voor me zorgt en me vertrouwen en energie geeft! Je bent een voorbeeld voor mij, zonder jou had ik dit niet gekund. Ik kijk er naar uit dat we binnenkort voor altijd aan elkaar verbonden worden.